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13. ABSTRACT ***(Maximum 200 Words)***

The progression of primary and metastatic prostate cancer depends upon the establishment of an adequate blood supply. The purpose of our ongoing research is to evaluate the relevance of angiogenesis in the pathogenesis of prostate cancer. We are evaluating the relationship of the expression of the angiogenesis factors bFGF, VEGF, and IL-8 with prostate cancer growth and metastasis, using our orthotopic model of metastatic prostate cancer in nude mice. Using sense and antisense technology we have enforced the expression of IL-8 by prostate cancer cells prior to their implantation into the prostate of nude mice and evaluated the causality between the expression of this angiogenic factor by human prostate cancer growing in nude mice, tumorigenesis, tumor induced angiogenesis, and metastasis. Similar studies with bFGF and VEGF are in progress. Once the relationship between angiogenesis factor expression and tumor virulence is established, we will target the expression of these factors by human prostate cancer established in nude mice with antisense gene therapy. To complement these studies we are evaluating the expression of VEGF, IL-8, and bFGF by human prostate cancer to determine their relationship to progression in patients. Increasing VEGF mRNA levels are highly associated with advancing pathologic stage.

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Introduction

The purpose of this research is to evaluate the relevance of angiogenesis in the pathogenesis of human prostate cancer. We are evaluating the relationship of the expression of the angiogenesis factors bFGF, VEGF, and IL-8 with prostate cancer growth and metastasis, using our orthotopic models of metastatic prostate cancer in athymic nude mice. Using sense and antisense technology we will enforce the expression of these factors by prostate cancer cells prior to their implantation into the prostate of nude mice and evaluate the causality between the expression of these angiogenesis factors by human prostate cancer growing within the prostate of athymic nude mice, and tumorigenesis, tumor induced angiogenesis, and metastasis. Once the relationship between angiogenesis factor expression and tumor virulence is established, we will target the expression of these factors by human prostate cancer established in nude mice with antisense gene therapy. To complement these studies we are evaluating the expression of VEGF, IL-8, and bFGF by human prostate cancer, and are evaluating VEGF expression as a molecular staging marker for patients with localized prostate cancer prior to radical prostatectomy.

Task 1

Task 1 was designed to evaluate the in vitro expression of the angiogenic factors VEGF, IL-8, and bFGF by prostate cancer cells from the same lineage which have differing metastatic properties. The hypothesis was that the expression of these factors would correlate with in vivo growth. The human prostate cancer cells LNCaP and the derived cell lines LNCaP-Pro5, and LNCaP-LN3 were cultured in vitro and then implanted into the prostates of athymic nude mice[1]. Prior to implantation we confirmed that the highly metastatic derived line LNCaP-LN3 overexpressed VEGF compared to either the parental LNCaP which was intermediate in its expression, and the line selected for growth within the prostate, LNCaP-Pro 5, expressed the lowest levels of VEGF at both the mRNA and protein level. These results were published in Clinical Cancer Research in April 1999 (See Appendix 1, Page 17, Ref. 2). We also evaluated the expression of VEGF, bFGF, and IL-8 by PC-3 and the highly metastatic PCM-LN4 cell line. We observed that PC-3M-LN4 overexpressed IL-8 and bFGF relative to PC3-P (Appendix 2, Page 37, Ref. 3). Neither cell line expressed significant levels of VEGF. Therefore, the in vitro expression of VEGF by the LNCaP lineage and IL-8 and bFGF by the PC-3 lineage correlated directly with their in vivo growth potential (Appendices 1 and 2).

Task 2

Task 2 was designed to evaluate the in vivo expression of VEGF, IL-8, and bFGF by the human prostate cancer cells growing within the prostates of athymic nude mice, and to correlate this expression with tumorigenicity, tumor induced angiogenesis, and metastasis. We published our results which showed that the highly metastatic LNCaP-LN3 cell line growing within the prostate of athymic nude mice overexpressed VEGF mRNA and protein compared with either LNCaP which had intermediate expression, and LNCaP-Pro 5, which expressed the lowest levels of VEGF[2]. Furthermore by anti-CD31 immunostaining the LNCaP-LN3 tumors were more vascular than either LNCaP (intermediate) or LNCaP-Pro5 (least vascular)[2]. Thus the in vivo expression of these factors correlated with in vitro expression and tumor induced angiogenesis and metastasis (See Appendix 1). Serum levels of VEGF were consistent with gene and protein expression. We evaluated the in vivo expression of IL-8, bFGF, and VEGF mRNA and protein by PC3-P and PC-3M-LN4 growing within the prostate of athymic nude mice. The highly metastatic PC-3M-LN4 cell line overexpressed IL-8 and bFGF mRNA and protein relative to the poorly metastatic PC3P cell line. By anti-CD31 immunostaining the PC3P-LN4 tumors were more vascular than the PC3P tumors (See Appendix 2, Page 19). These studies confirm that the in vivo expression of these angiogenic factors mirrors their in vitro expression, and that the overexpression of these factors correlates with enhanced tumor induced neovascularization and metastatic potential.

As an extension of this task we demonstrated the causality between IL-8 expression and the tumorigenicity and metastatic potential of human prostate cancer. We transfected the poorly metastatic prostate cancer cell line PC3-P with sense IL-8 vectors and the highly metastatic PC-3M-LN4 cell line with antisense IL-8 constructs. After determining that the IL-8 expression by PC3-P was increased and the expression by PC-3M-LN4 was decreased these cells were implanted into the prostates of athymic nude mice. Sense transfected PC3-P cells demonstrated increased IL-8 expression, and enhanced tumorigenicity, tumor induced neovascularization, and metastasis compared with Neo transfected controls and PC3-P. Conversely, antisense transfected PC-3M-LN4 demonstrated decreased IL-8 expression and decreased tumorigenicity, tumor induced angiogenesis, and metastasis compared with Neo controls or PC-3M-LN4. Moreover, we also demonstrated that IL-8 regulates MMP-9 expression by human prostate cancer (See Appendix 2).

Task 3

The sense and antisense adenoviral vectors for bFGF, VEGF, and IL-8 have been constructed. The bFGF sense and antisense vectors were tested in human Transitional Cell Carcinoma (TCC) lines. The human bladder cancer cell lines 253J and 253J B-V were infected with recombinant adenovirus overnight, at MOI's of 1:1, 1:3, 1:5, 1:10, 1:20, 1:50, and 1:100. A β-galactosidase adenovirus was used as a control for these experiments (See Appendix 3 for vector construction). Cytotoxicity was assessed through cell counting 48-72 hours after infection. Expression of cell associated and secreted bFGF was determined by ELISA assay of cell lysates and conditioned media from each MOI. An MOI of 10:1 was shown to be optimal for both Ad5 XCMV bFGF and Ad5 XCMV bFGF AS, demonstrating minimal cytotoxicity and maximal effect on bFGF production. The optimal MOI for the β-galactosidase adenovirus was found to be 5:1, demonstrating no effect on bFGF production, evidence of β-galactosidase production (data not shown), and minimal cytotoxicity. These studies demonstrate the feasibility of sense and antisense infection.

Task 4

Utilizing Ad5PCA14 sense and anti sense vectors (sense= Ad5PCA14VEGF, anti sense = Ad5PCA14 αVEGF) an assessment of cell viability and VEGF modulation was performed using LNCaP parental cells (appendices 4-7). LNCaP viability was excellent at MOI from 1:1- 1:20 with overexpression of VEGF first noted at MOI of 1:5 (using the Ad5PCA14VEGF vector, appendices 4-5). In contrast, VEGF was significantly downregulated at MOI of 1:5 while cell viability was maintained up to MOI of 1:20 using the Ad5PCA14 αVEGF vector (Appendices 6-7). Similarly PC-3M-LN4 cells were infected with adenoviral IL-8 antisense at several MOI's (1:1- 100:1) and IL-8 protein was measured and compared with a control vector. As shown in appendix 8, IL-8 secretion was specifically inhibited by the antisense vector.

Tasks 5&6 (Pending)

Studies to determine the invivo effect of adenoviral mediated modulation ofIL-8, bFGF, and VEGF on PC-3 and LNCaP cell lineages will be carried out in the coming year. Data from the above transfection studies strongly suggests that IL-8 is

directly involved in the regulation of metastasis. Treatment of tumor bearing mice utilizing antisense strategies against the relevant angiogenic targets will follow.

Task 7

VEGF, bFGF, and IL-8 are angiogenic factors known to be expressed in prostate cancer (4-11). Recently we compared the expression of VEGF by in situ hybridization (ISH) in 34 radical prostatectomy specimens to determine the relationship of VEGF expression to tumor location ,Gleason score , and pathological stage (Appendix 9, Ref. 11). VEGF expression did not differ significantly within a given tumor but was significantly higher in tumors of higher Gleason score and pathologic stage. In fact, VEGF expression by ISH was highly associated with seminal vesicle involvement and lymph node metastasis in radical prostatectomy specimens and this was independent of the Gleason score. In order to determine whether evaluation of VEGF expression could provide valuable staging information and identify patients with advanced disease prior to therapy , we have begun to correlate VEGF expression by ISH (as well as type IV collagenase and e-cadherin) in radical prostatectomy specimens with pre-therapy biopsies (Appendix 10). Our preliminary results indicate a statistically significant correlation (Kappa statistic) between biopsy and radical prostatectomy specimens. However, in some cases where the dominant tumor was not sampled in the biopsy, it was not predictive. Thus these preliminary results suggest that VEGF expression may serve as a useful molecular marker to improve the staging of patients with prostate cancer and that adequate sampling is necessary.

(7) Key Research Accomplishments

- Identified that highly metastatic prostate cancers overexpress the angiogenic factors VEGF, bFGF, and IL-8.
- Demonstrated the causality between IL-8 expression and tumorigenicity and metastasis of human prostate cancer which is therefore a potentially suitable therapeutic target for advanced prostate cancer
- Demonstrated that IL-8 regulates MMP-9 expression by human prostate cancer
- Demonstrated the feasibility of antisense adenoviral infection of human prostate cancer

•Determined that VEGF expression is associated with advanced pathologic stage and Gleason score in patient specimens. This serves as a rationale to further explore its role as a molecular marker for the staging and prognosis of human prostate cancer.

(8) Reportable Outcomes

Manuscripts	-Balbay manuscript published in Clin Can Res
	-Inoue manuscript (submitted)
	-Kuni manuscript (submitted)
Abstracts	-Dinney AUA 1998-VEGF
	-Pettaway AUA 1999-Molecular staging

(9) Conclusions

We have accumulated data which indicates that angiogenesis regulates the growth and metastasis of human prostate cancer. We have identified a cause and effect between IL-8 expression and metastasis. However given the heterogeneity of angiogenesis factor expression by human prostate cancer, it is important to identify whether the expression of other factors such as VEGF and bFGF also regulate this process. If that is the case these proteins become potential targets for therapy. Furthermore given that we can alter the expression of angiogenesis factor expression by prostate cancer with sense or antisense adenoviral constructs it is important to establish the feasibility of gene therapy targeting these factors.

Our preliminary data indicates that the expression of VEGF has prognostic value for predicting seminal vesical invasion or lymph node metastasis in patients with clinically localized prostate cancer. If this can be validated in a larger patient population then VEGF (bFGF or IL-8) may become a valuable molecular marker that could be used both to identify patients with advanced disease who require aggressive therapy, and serve as a target for that therapy.

In summary, the results of our experiments indicate the relevance of angiogenesis and the expression of angiogenesis factors in the pathogenesis of human prostate cancer. Angiogenesis factors regulate the growth and metastasis of prostate cancer, and also serve as novel targets for therapy. These factors aer also valuable staging markers, and could be used to modify and improve the selection of therapy for patients with prostate cancer.

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Highly Metastatic Human Prostate Cancer Growing within the Prostate of Athymic Mice Overexpresses Vascular Endothelial Growth Factor[1]

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ABSTRACT

Angiogenesis is essential for tumor progression and metastasis. It is mediated by the release of angiogenic factors by the tumor or host. We analyzed the expression of angiogenic factors by the prostate cancer cell line LNCaP and two derived variants, *in vitro* and *in vivo*, to determine whether metastatic cell lines express higher levels of these factors. The production of three angiogenic factors, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and interleukin 8 (IL-8), by LNCaP and its variants, LNCaP-LN3 (highly metastatic) and LNCaP-Pro5 (slightly metastatic), was measured by ELISA. VEGF, bFGF, and IL-8 mRNA expression was determined *in vitro* by Northern blot analysis. VEGF mRNA expression was determined *in vivo* by *in situ* hybridization. VEGF and flk-1 protein expression and microvessel density of LNCaP cell tumors were quantified by immunohistochemistry. *In vitro*, VEGF production by LNCaP-LN3 (3.15 ± 0.04 pg/ml/10^3 cells) was significantly higher than those of both LNCaP (2.38 ± 0.34 pg/ml/10^3 cells) and LNCaP-Pro5 (1.67 ± 0.37 pg/ml/10^3 cells; $P = 0.049$ and 0.001, respectively). None of the three cell lines produced detectable levels of bFGF or IL-8 *in vitro*. *In vivo*, LNCaP-LN3 tumors exhibited higher levels of VEGF mRNA and protein (152.2 ± 28.5 and 200.5 ± 28.3) and of flk-1 protein (156.5 ± 20.6) and had higher microvessel density (16.4 ± 4.2) than either LNCaP tumors (89 ± 17.5, 173.3 ± 23.0, 124.6 ± 21.6, and 12.4 ± 3.5, respectively) or LNCaP-Pro5 tumors (63 ± 14.7, 141.2 ± 38.1, 126.1 ± 20, and 5.8 ± 2.2, respectively). In conclusion, metastatic human prostate cancer cells exhibited enhanced VEGF production and tumor vascularity compared with prostate cancer cells of lower metastatic potential. Thus, VEGF may play an important role in prostate cancer metastasis.

INTRODUCTION

Tumor growth and metastasis depend upon the induction of a blood supply (1, 2). This process, angiogenesis, is regulated by a diverse group of molecules, including VEGF[3] (2, 3), bFGF (4, 5), and IL-8 (6). The prevascular phase of a tumor is usually associated with local, nonmetastatic tumors; a vascular phase precedes invasion and metastasis (7). The vascular density of prostate cancer, a histological indicator of angiogenesis, correlates with invasion and metastasis (8, 9).

The acquisition of an angiogenic phenotype is mediated by angiogenic factors released by the tumor or host cells and depends upon the balance between stimulatory and inhibitory factors released by the tumor and its microenvironment (10). Following the induction of vascularization, the rate of tumor growth increases exponentially (11, 12).

The specific angiogenic factors regulating prostate cancer growth and metastasis have not been elucidated. It is highly unlikely that any one factor will be solely responsible for angiogenesis in all prostate cancers, and furthermore, multiple factors may be necessary for angiogenesis to occur in a single tumor.

VEGF is expressed by both benign and malignant prostate cells as well as by neuroendocrine cells (13). The level of expression by malignant cells is greater than that by benign prostate cells (14, 15). VEGF expression enhances the tumorigenicity of human prostate cancer. Administration of VEGF to mice receiving whole-body irradiation increased the growth of human prostate cancer xenografts and led to rapid tumor progression (16). In addition, administration of an anti-VEGF antibody to nude mice that were growing the human prostate cancer cell line DU145 completely inhibited neovascularization within the tumor (17). In androgen-responsive cells, VEGF expression seems to be androgen dependent. For instance, VEGF expression by the androgen-responsive PC-82 and A-2 human prostate lines growing s.c. in severe combined immunodeficient mice was inhibited by castration (18). Androgen withdrawal also down-regulates VEGF production by human prostate cancer (19, 20).

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL-8, interleukin 8; GAPDH, glyceraldehyde phosphate dehydrogenase; IHC, immunohistochemistry; ISH, *in situ* hybridization.

Table 1 Tumorigenicity and production of metastasis by LNCaP cells and two variants subsequent to orthotopic implantation in atymic nude mice

LNCaP cell line	Tumorigenicity	Prostate weight, g (mean ± SD)	Para-aortic lymph node metastases
LNCaP	24/43	1.2 ± 1.0	12/43
LNCaP-Pro5	10/17	3.3 ± 1.7[a]	2/17
LNCaP-LN3	19/19[b]	1.2 ± 0.9	13/19[c]

[a] $P < 0.05$, compared with LNCaP and LNCaP-LN3.

[b] $P < 0.001$, compared with LNCaP: $P = 0.002$, compared with LNCaP-Pr05.

[c] $P < 0.003$, compared with LNCaP: $P < 0.001$, compared with LNCaP-Pr05. Adapted from Pettaway *et al.* (21).

Here, we evaluated the expression of angiogenic factors in an orthotopic model of human prostate cancer. We previously established this model by directly implanting the human prostate cancer cell line LNCaP into the prostates of athymic nude mice. We selected from the parental LNCaP cell line distinct subpopulations that either were more tumorigenic within the prostate (LNCaP-Pro5) or had a greater propensity to metastasize (LNCaP-LN3: Ref. 21). The purpose of the study described herein was to evaluate whether the expression of the angiogenic factors VEGF, bFGF, and IL-8 by LNCaP and its variants, LNCaP-Pro5 and LNCaP-LN3, correlated with enhanced angiogenesis and metastasis.

MATERIALS AND METHODS

Tumor Cell Lines. The three cancer cell lines were maintained as monolayers in RPMI 1640 supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin. The low metastatic LNCaP-Pro5 and high metastatic LNCaP-LN3 variants were isolated by intraprostatic injection of LNCaP and sequential selection for nonmetastatic and metastatic variants as described previously. The LNCaP cell line is intermediate in its metastatic potential compared with these variant lines (Table 1; Ref. 21).

Animals. Male athymic BALB/c nude mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a laminar air flow cabinet under specific pathogen-free conditions and used at 8–12 weeks of age. Animal facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the standards of the United States Department of Agriculture, Department of Health and Human Services, and IH.

ELISA. Fifty thousand viable cells from the LNCaP, LNCaP-Pro5, and LNCaP-LN3 cell lines were seeded onto 35-mm Petri dishes. Conditioned medium was removed after 9 h of incubation and centrifuged at 5000 rpm for 5 min before washing with 1 ml of HBSS. Both conditioned medium and cell suspensions were stored at −20°C prior to assay. The levels of cell-associated and supernatant VEGF, bFGF, and IL-8 were measured using commercially available ELISA kits (Quantikine; R&D Systems, Minneapolis, MN). The protein level for each angiogenic factor was quantified by comparing its optical density to the standard curve for each factor and normalizing for cell number.

Northern Blot Analysis. Polyadenylated mRNA was extracted from 50–70% confluent monolayer cultures of cells growing in culture using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). The mRNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel and electrotransferred to a GeneScreen nylon membrane (DuPont, Boston, MA) using a UV cross-linker (Stratalinker, model 1800; Stratagene, La Jolla, CA) cross-linked with 120,000 μJ/cm^2. Filters were washed at 55°C with 30 mM sodium citrate and 0.1% sodium dodecyl sulfate (w/v). The membranes were then hybridized and probed for VEGF, bFGF, and IL-8; GAPDH was used as a control for loading. The cDNA probes used were (*a*) a 1.3-kb *Pst*I cDNA for GAPDH (22); (*b*) a 1.4-kb cDNA fragment of bovine bFGF (23); (*c*) a 204-bp *Bam*HI-*Eco*RI fragment of the human VEGF cDNA (a gift of Dr. Brygida Berse, Harvard Medical School, Boston, MA; Ref. 24); and (*d*) a 0.5-kb *Eco*RI cDNA fragment corresponding to human IL-8 (a gift of Dr. K. Matsushima, Kanazawa, Japan; Ref. 25). The probes were radiolabeled by a random primer technique and [α-^{32}P]dCTP (Amersham Corp.). Autoradiography of the membrane was performed after washing. Densitometry scanning permitted quantification of the bands.

Orthotopic Implantation of the Tumor Cells. For the *in vivo* portion of the study, cultured LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells (70% confluent) were prepared for injection as described previously (21). Mice were anesthetized with methoxyflurane, a lower midline incision was made, and the prostate was exposed. Viable tumor cells (2×10^6 cells in 40 μl of HBSS) were injected into one of the dorsal lobes of the prostate. The formation of a "bleb" was the sign of a satisfactory injection. Organs were returned to their proper locations, and the abdominal wall was closed in a single layer with metal clips.

Necropsy. The mice were killed 5 weeks after injection. Prostate tumors were harvested, weighed, and either embedded in OCT solution (Sokera Inc., Torrence, CA) for frozen sections or fixed in formalin for paraffin sections.

Immunohistochemical Determination of VEGF, bFGF, IL-8, and flk-1. The expression of VEGF, bFGF, IL-8, and VEGF receptor flk-1 was detected in paraffin sections of tumors using rabbit polyclonal IgG antihuman antibodies for VEGF, bFGF, and IL-8 diluted to 1:500, 1:500, and 1:50, respectively. An antimouse polyclonal antibody to flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:100 dilution. The α-immunoperoxidase technique for IHC staining was performed with a second peroxidase-conjugated goat antirabbit antibody (IgG, F[ab]$_2$ fragment; Jackson ImmunoResearch Laboratory, West Grove, PA) at a 1:500 dilution. We confirmed the specificity of the VEGF and flk-1 staining by the absorption test using the control peptides SC 152P and SC 315P. Briefly, primary antibodies were pretreated with control peptide overnight at 4°C, and this pretreated antibody was used for IHC analysis, as described above. No immunostaining was observed using the pretreated antibodies (data not shown).

Quantification of Microvessel Density. Cryostat sections of tumors were fixed with 2% paraformaldehyde in PBS (pH 7.5) for 10 min and then washed twice with PBS. The sections were then treated for 5 min with 1% Triton X-100 and

Table 2 In vitro production of bFGF, IL-8, and VEGF in LNCaP cells and selected variants

bFGF, IL-8, and VEGF concentrations (pg/ml/10^3 cells) were measured in both cell-associated and culture supernatants of LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells after 96 h. Values are means ± SD of four separate experiments.

	Culture supernatants			Cell-associated supernatants		
Cell line	bFGF	IL-8	VEGF[a]	bFGF	IL-8	VEGF
LNCaP	0	0	2.38 ± 0.34	0	0	0
LNCaP-Pro5	0	0	1.67 ± 0.37	0	0	0
LNCaP-LN3	0	0	3.15 ± 0.04	0	0	0.25 ± 0.02
253JB-V	0	8.50 ± 3.24	2.87 ± 0.05	2.77 ± 0.87	1.76 ± 0.06	0

[a] Tukey's honestly significant difference test *Ps*: LNCaP *vs.* LNCaP-Pro5, $P = 0.064$; LNCaP-LN3 *vs.* LNCaP, $P = 0.049$; LNCaP-LN3 *vs.* LNCaP-Pro5, $P = 0.001$.

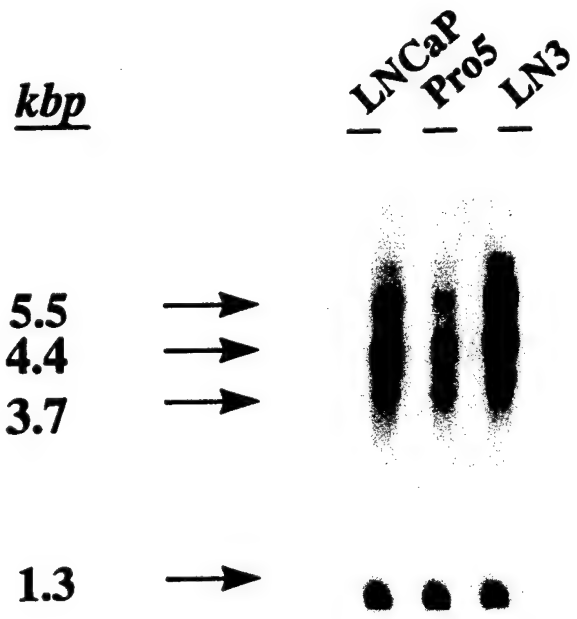

Fig. 1 In vitro VEGF expression in LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells, as shown by Northern blot analysis. Relative VEGF mRNA expression was highest in LNCaP-LN3 (1.64) compared with LNCaP-Pro5 (0.41) and LNCaP (1.09) after normalization to GADPH. Four distinct mRNA transcripts were identified in the LNCaP cell lines.

washed three times with PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol, and the sections were washed with PBS and incubated overnight in protein-blocking solution. The excess blocking solution was removed, and the samples were incubated with rat antimouse CD31 antibody that stains endothelial cells (PharMingen, San Diego, CA). Swine peroxidase-conjugated anti-rabbit antibody was applied for 30 min after the primary antibody was removed. The samples were rinsed with PBS and developed with 3-amino-9-ethylcarbazole at room temperature for 20 min. The sections were counterstained with aqueous hematoxylin. A positive reaction was indicated by a brownish precipitate.

The tissues were examined at low power (×40), and five high-power fields of viable tumor at the periphery of the tumor were selected for vessel counts. Selected fields (high-power field, ×20 objective and ×10 ocular, 0.739 mm^2 per field) were recorded using a computer-linked cooled CCD Optronics Tec 470 camera (Optronics Engineering, Goletha, CA). Microvessels were quantified according to the method described by Weidner *et al.* (26). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The results were expressed as the number of microvessels identified within a single ×200 field.

Oligonucleotide Probes. Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts of the three angiogenesis-related genes based on the published reports of the DNA sequence: VEGF/vascular permeability factor, TGG'TGA'TGT'TGG'ACT'CCT'CAG'TGG'GCU, 57.7% guanosine-cytosine content (24); bFGF, CGG'GAA'GGC'GCC'GCT'GCC'GCC', 85.7% guanosine-cytosine content (23); IL-8, CTC'CAC'CCA'CCT'CTG'CAC'CC), 65% guanosine-cytosine content (25). The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCG; Madison, WI) based on the FastA algorithm (27). These sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis. A poly$(dT)_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling using standard phosphoramidile chemistry (Research Genetics, Huntsville, AL; Ref. 28). The lyophilized probes were reconstituted to a stock solution at 1 μg/μl in 10 mM Tris (pH 7.6) and 1 mM EDTA. The stock solution was diluted with Probe Diluent (Research Genetics) immediately before use.

***In Situ* mRNA Hybridization.** Paraffin-embedded sections of fixed tissue (3–5 μm) were mounted on ProbOn slides (Fisher Scientific, Pittsburgh, PA). The slides were dewaxed and prepared, and ISH was performed using the MicroProbe system (Fisher Scientific) as described previously (29, 30). Slides were rinsed three times in Tris buffer for 30 s; the probes were then hybridized at 45°C for 45 min. The slides were washed three times for 2 min each time with 2× SSC at 45°C. The samples were then incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), and then briefly (1 min) rinsed in alkaline phosphatase enhancer.

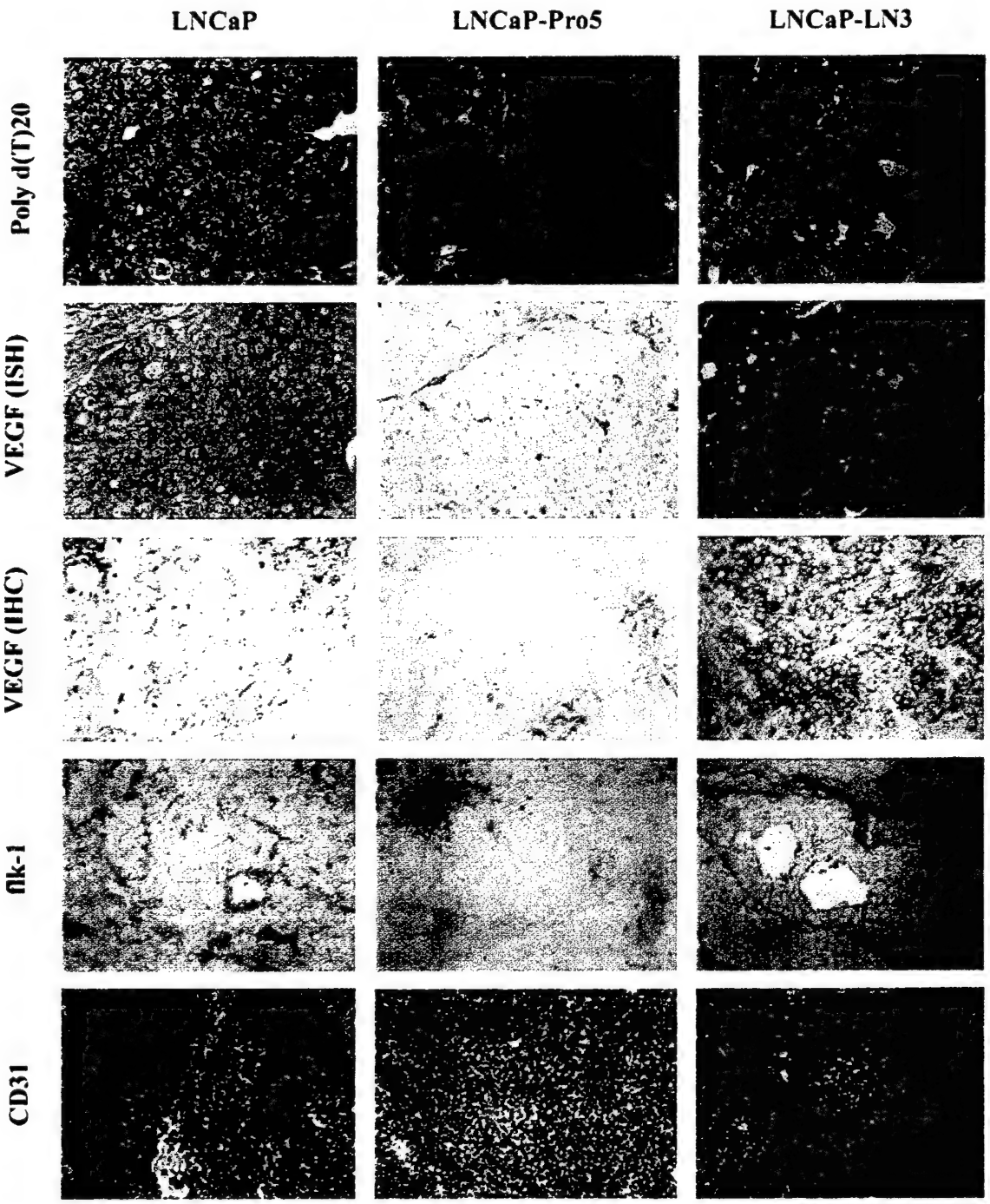

Fig. 2 ISH and immunohistochemical staining of LNCaP, LNCaP-Pro5, and LNCaP-LN3 growing in the prostates of athymic nude mice. Note the increased expression of both VEGF mRNA and protein in the highly metastatic LNCaP-LN3 tumors. Poly(dT) staining served as the control for mRNA integrity. Microvessel density by anti-CD31 staining of endothelial cells and flk-1 expression on tumoral vessels were also greater in LNCaP-LN3 cell tumors.

The samples were then incubated with chromogen substrate for 20 min at 45°C. If necessary, an additional incubation was performed with fresh chromogen to enhance a weak reaction. The samples were then covered with Universal Mount mounting medium (Research Genetics), heat-dried, and examined. Red staining indicated a positive reaction in this assay. Appropriate controls for endogenous alkaline phosphatase were included by treating the samples in the absence of biotinylated probe, using chromogen alone. No immunoreactivity was observed in the controls.

Table 3 *In situ* mRNA and immunohistochemical analysis of VEGF, VEGF receptor flk-1, and microvessel density in LNCaP and selected variants

Mice were injected with 2×10^6 cells and necropsied 5 weeks after injection. Five different areas of three separate tumors were analyzed. Values are means ± SD of 15 determinations of each cell line. Statistical comparisons were performed using Tukey's honestly significant difference test.

Cell line	VEGF (ISH)[a]	VEGF (IHC)[b]	flk-1[c]	CD31[d]
LNCaP	89 ± 17.5	173.3 ± 23.0	124.6 ± 21.6	12.4 ± 3.5
LNCaP-Pro5	63 ± 14.7	141.2 ± 38.1	126.1 ± 20.0	5.8 ± 2.2
LNCaP-LN3	152.2 ± 28.5	200.5 ± 28.3	156.5 ± 20.6	16.4 ± 4.2
Tukey's honestly significant difference test *P*s				
LNCaP *vs.* LNCaP-Pro5	0.117	0.011	0.973	0.001
LNCaP *vs.* LNCaP-LN3	0.001	0.023	0.001	0.001
LNCaP-LN3 *vs.* LNCaP-Pro5	0.001	0.001	0.001	0.001

[a] VEGF mRNA density was evaluated by computer-assisted image analysis and is expressed as the ratio of the intensity of tumor ISH to that of the normal glandular epithelium, normalized to poly(dT) expression. The differences between LNCaP and LNCaP-LN3 ($P = 0.001$) and between LNCaP-LN3 and LNCaP-Pro5 ($P = 0.001$) were significant.

[b] VEGF cytoplasmic staining was evaluated by computer-assisted image analysis and is expressed as a ratio of the tumor expression to normal prostatic glandular epithelial cell expression. The differences between LNCaP and LNCaP-Pro5 ($P = 0.011$), between LNCaP and LNCaP-LN3 ($P = 0.023$), and between LNCaP-LN3 and LNCaP-Pro5 ($P = 0.001$) were significant.

[c] flk-1 cytoplasmic staining in endothelial cells was evaluated by computer-assisted image analysis and is expressed as a ratio of tumor vascular endothelium expression to normal glandular vascular endothelium expression. The differences between LNCaP and LNCaP-LN3 and between LNCaP-LN3 and LNCaP-Pro5 were significant ($P = 0.0001$ for both comparisons).

[d] CD31 staining reflecting microvessel density was counted under ×200 magnification in 0.739-mm^2 fields. The differences between LNCaP and LNCaP-Pro5, between LNCaP and LNCaP-LN3, and between LNCaP-LN3 and LNCaP-Pro5 were significant ($P = 0.0001$ for each comparison).

To check the specificity of the hybridization signal, the following controls were used: (*a*) RNase pretreatment of tissue sections, (*b*) substitution of the antisense probe with a biotinylated sense probe, and (*c*) competition assay with unlabeled antisense probes. Markedly decreased or no signal was obtained after all of these treatments (31).

Densitometry Quantification of IHC and ISH. The intensity of IHC staining and ISH was evaluated in five fields at the periphery of the tumors representing areas of most intense staining. Each field was evaluated using the ImageQuant analyzer and Optimas software program (Bioscan. Edmonds, WA). IHC staining intensity of each sample was compared with the staining intensity of the normal prostate glands in the sample and expressed as a ratio (tumor cells:normal glandular cells). ISH was quantified in a similar manner using serial sections of the same tumor block. Normal prostate glands served as the internal control for mRNA expression, and poly(dT) staining controlled for mRNA preservation. Results were expressed as the ratio of the intensity of tumor ISH staining to that of normal glandular cell staining and normalized for poly(dT) expression.

Statistical Analysis. One-way ANOVA was used to examine group differences in VEGF production by the cell lines *in vitro* and in VEGF mRNA and protein expression as well as flk-1 protein expression on tumor sections *in vivo*. When ANOVA indicated a significant ($P < 0.05$) difference, a post hoc test was performed using Tukey's honestly significant difference multiple comparison test.

RESULTS

***In Vitro* Expression of VEGF, bFGF, and IL-8.** The *in vitro* production of cell-associated and secreted VEGF, IL-8, and bFGF protein by the parental LNCaP cell line and the variant LNCaP-Pro5 and LNCaP-LN3 cell lines is shown in Table 2. The 253J B-V cell line served as a positive control for bFGF and IL-8 production (32). By ELISA, VEGF was the only angiogenic peptide detected among the LNCaP cell lines. The highly metastatic LNCaP-LN3 cell line secreted significantly more VEGF (3.15 ± 0.04 pg/ml/10^3 cells) than the LNCaP cell line (2.38 ± 0.34 pg/ml/10^3 cells) or the LNCaP-Pro5 line (1.67 ± 0.37 pg/ml/10^3 cells; $P = 0.049$ and 0.001, respectively).

Northern blot analysis confirmed the results of ELISA. Densitometric analysis indicated that the relative steady-state gene expression of VEGF by LNCaP-LN3 was 4-fold greater than that by LNCaP-Pro5 and 1.5-fold greater than that by LNCaP (Fig. 1). Four VEGF transcripts were identified by Northern blot analysis. We did not identify bFGF or IL-8 mRNA transcripts in the three LNCaP cell lines (data not shown).

***In Vivo* Expression of VEGF, bFGF, IL-8, flk-1, and Microvessel Density.** We used IHC and ISH to compare the expressions of VEGF, bFGF, IL-8, and flk-1 and the microvessel densities of the LNCaP, LNCaP-Pro5, and LNCaP-LN3 cell lines growing in the prostates of nude mice. Representative tumor sections analyzed for immunoreactivity of VEGF are shown in Fig. 2. The immunoreactivity of VEGF was highest in the LNCaP-LN3 tumors, intermediate in LNCaP tumors, and lowest in LNCaP-Pro5 tumors, which is in accordance with our *in vitro* findings. Computer-assisted analysis of representative sections confirmed these results (Table 3). Using ISH, we observed that steady-state mRNA expression of VEGF was also greatest in the LNCaP-LN3 tumors, intermediate in the LNCaP tumors, and least in the LNCaP-Pro5 tumors (Fig. 2). Relative expressions of VEGF mRNA and protein were 2.4-fold higher in the LNCaP-LN3 tumors than in the LNCaP-Pro5 tumors (Fig. 2 and Table 3). *In vivo* bFGF and IL-8 protein (by IHC) and mRNA (by ISH) were expressed at similar low levels in all three tumors (data not shown).

By anti-CD31 immunostaining, microvessel density was significantly greater in the LNCaP-LN3 tumors than in either LNCaP or LNCaP-Pro5 tumor tissue ($P = 0.001$; Fig. 2 and Table 3). By image analysis, the VEGF receptor flk-1 was also significantly overexpressed on endothelial cells within the

LNCaP-LN3 tumors compared with either LNCaP or LNCaP-Pro5 tumors (Fig. 2 and Table 3).

DISCUSSION

Tumor growth and metastasis depend upon the induction of a blood supply. This process of angiogenesis is mediated, in part, by the secretion of angiogenic factors such as VEGF by tumors growing in their relevant microenvironment. VEGF is secreted by a wide variety of tumor cells, and its secretion correlates with the metastatic potential of these tumor cells (33–35). Our data indicate that the malignant potential of the human prostate carcinoma cell line LNCaP and its variant lines, LNCaP-LN3 and LNCaP-Pro5, correlated with their VEGF expression. *In vitro* evaluation revealed that the highly metastatic cell line LNCaP-LN3, which we selected for its ability to metastasize to regional lymph nodes, overexpressed VEGF protein compared with the poorly metastatic cell line LNCaP-Pro5, which was selected for its growth within the prostate. Increased steady-state gene expression of VEGF mRNA was also observed in the LNCaP-LN3 cell line. By Northern blot analysis, we observed four mRNA transcripts. The relative expressions of these four VEGF mRNA transcripts by the three cell lines were compared following normalization to GADPH. These transcripts represent both secreted and freely soluble VEGF (36). The increased expression of VEGF by LNCaP-LN3 was confirmed *in vivo* using IHC and ISH. Assessment of VEGF mRNA and protein was performed at the invasive edge of each tumor because the centers of the tumors were often necrotic (and potentially hypoxic and acidotic), making VEGF expression within these regions difficult to interpret because of artifacts (37, 38). Using antibodies directed against murine CD31, we also identified increased microvessel density in the periphery of the LNCaP-LN3 prostate tumor, which corresponded to the area of greatest VEGF expression. This suggests that the local production of VEGF by metastatic prostate cancer cells induced the increase in neovascularization, which presumably resulted in enhanced metastasis. VEGF is likely an important angiogenic factor secreted by LNCaP cells because we did not detect significant expression of either bFGF or IL-8 by these cells *in vitro* or *in vivo*. However, other angiogenic molecules, such as platelet-derived endothelial cell growth factor, transforming growth factor-β, and angiogenin, were not measured in this study.

VEGF promotes the formation of new capillaries by stimulating endothelial cell division and migration and by increasing capillary permeability following ligand binding to the endothelial cell surface membrane (39–41). We observed that the VEGF receptor protein flk was overexpressed within all LNCaP tumors compared with normal prostate epithelium (data not shown). In addition, the intensity of immunoreactivity to anti-flk-1 was increased on the cell membrane of the endothelial cells within LNCaP-LN3 tumors compared with either LNCaP or LNCaP-Pro5, suggesting that VEGF up-regulates its own receptors on the endothelial cells within the tumor. These observations are consistent with previous reports in which the VEGF receptor protein flk was up-regulated in gastrointestinal tract adenocarcinomas (33), renal cell carcinoma (34, 42), and transitional cell carcinoma of the bladder (42).

In conclusion, these results indicate that the metastatic potential of a human prostate cancer cell line correlates with its VEGF expression. We further speculate that VEGF plays a role in spontaneous metastasis subsequent to orthotopic implantation of LNCaP cells. Studies are currently in progress to directly test this hypothesis.

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Interleukin-8 Expression Regulates Metastases in Androgen Independent Prostate Cancer

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25 Text pages; 9 Figures; ____Tables.

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The abbreviations used are: IL-8, interleukin-8; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial cell growth factor; MMP-9, matrix metalloproteinase type 9; CM, conditioned medium; CMEM, complete Eagle's minimum essential medium; ELISA, enzyme-linked immunosorbent assay; ISH, in situ hybridization; IHC, immunohistochemical staining; MTT, methyethiotetrazole; CaP, prostatic adenocarcinoma.

Abstract

Interleukin-8 is mitogenic and chemotactic for endothelial cells. Within a neoplasm, interleukin-8 is secreted by inflammatory and neoplastic cells. The highly metastatic PC3M-LN4 cell line overexpresses interleukin-8 relative to the poorly metastatic PC3-P cell line. We evaluated whether interleukin-8 expression by human prostate cancer growing within the prostate of athymic nude mice regulates tumor angiogenesis, growth, and metastasis. PC3-P cells were transfected with the full length sense IL-8 cDNA, while PC3M-LN4 cells were transfected with the full-sequence antisense interleukin-8 cDNA. Control cells were transfected with the neomycin resistance gene (Neo). In vitro, sense-transfected PC3-P cells overexpressed interleukin-8 specific mRNA and protein which resulted in upregulation of matrix metalloproteinase 9 (MMP-9) mRNA, and collagenase activity, resulting in increased invasion through Matrigel. Following antisense transfection of the PC3M-LN4 cells, interleukin-8 and MMP-9 expression, collagenase activity, and invasion were markedly reduced relative to controls. Following orthotopic implantation, the sense-transfected PC3-P cells were highly tumorigenic and metastatic, with significantly increased neovascularity and interleukin-8 expression compared with either PC3-P cells or controls. Antisense transfection significantly reduced the expression of interleukin-8 and MMP-9 and tumor-induced neovascularity, resulting in inhibition of tumorigenicity and metastasis.

These results demonstrate that interleukin-8 expression regulates angiogenesis in prostate cancer, in part by induction of MMP expression, and subsequently regulates the growth and metastasis of human prostate cancer

Introduction

Prostate cancer is the most common malignancy and the second leading cause of cancer-related deaths among men in the United States.[1] Although modest improvements in early detection and therapy, have occurred, [2, 3] most deaths from prostate cancer are caused by metastases that resist conventional androgen-deprivation therapy.[4-6] Continued empiricism in the treatment of advanced prostate cancer is unlikely to produce significant improvement over current therapy. Rather, a knowledge of the cellular and molecular properties of prostate cancer and of the tumor-host

interactions that influence the dissemination of metastatic disease is essential for the design of more effective treatment.

Metastasis is a highly selective process involving multiple tumor-host interactions.[7-11] A crucial step in metastasis is vascularization in and around the tumor.[12, 13] This process of angiogenesis[14-16] is regulated by the balance between stimulatory and inhibitory factors released by the tumor and the microenvironment. [14-16] Human prostate cancer produces a number of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), [17, 18] basic fibroblast growth factor (bFGF), [19, 20] and interleukin 8 (IL-8). [18,21] Microvessel density (MVD), a pathological surrogate for angiogenesis, correlates with stage and prognosis for patients with prostate cancer.[22] IL-8 was originally identified as a leukocyte chemoattractant[23,24] but is now also known to be an autocrine growth factor for malignant melanoma[25] and keratinocytes.[26] In addition, IL-8 displays mitogenic and morphogenic activity for endothelial cells[27] and regulates angiogenesis in lung cancer[28,29] and melanoma.[30,31] While IL-8 is expressed by prostate cancer, and this expression correlates with metastatic potential,[31] the exact role of IL-8 in the process of prostate cancer growth, angiogenesis, and metastasis is unclear.

Therefore, in the present study, we forced the expression of IL-8 by human prostate cancer cells to determine whether IL-8 expression regulates angiogenesis and the subsequent tumorigenicity and metastasis of human prostate cancer growing within the prostates of athymic nude mice.

Materials and Methods

Cell Lines and Culture Conditions. Cells of the highly metastatic human prostate carcinoma cells line PC3M-LN4 and the poorly metastatic cell line PC3-P were grown as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (complete RPMI [CRPMI])[32].

Transfection and Selection of PC3-P and PC3M-LN4 Cells Expressing IL-8. Tumor cells were plated onto 100-mm dishes at a density of 1×10^6/dish. The monolayers (60% to 70% confluent) were transfected with pcDNA3/sense IL-8, pcDNA3/antisense IL-8 (a gift from Dr. K.

Matsushima[23]), or control pcDNA3/neo plasmids using a stable mammalian transfection kit from Strategene (La Jolla, CA). The cultures were placed in a 37°C incubator for 12 hours and then washed and fed with modified complete minimum essential medium (CMEM). After 24 hours, 500-1000 μg/ml G418 sulfate (Life Technologies, Inc. [Gibco BRL], Gaithersburg, MD) was added. The CMEM/G418 medium was replaced every 3 days until individual resistant colonies were isolated and established in culture as individual lines. All of the lines were maintained in CMEM/G418 and frozen after one to three *in vitro* passages. To avoid clonal variations, positive clones were then pooled for the *in vitro* and *in vivo* studies.

The poorly tumorigenic, poorly metastatic PC3-P cells and the highly tumorigenic, highly metastatic PC3M-LN4 cells were transfected with pcDNA3/sense IL-8, and pcDNA3/antisense IL-8, respectively, or with control pcDNA3/neo. Individual G418-resistant (500-1000 μg/ml) colonies were established as separate adherent cultures. We selected pooled sense IL-8 transfected PC3-P cells, PC3-P(IL-8), the highest IL-8 expressing clone, PC3-P(IL-8 High), and the lowest IL-8 expressing clone, PC3-P(IL-8 Low), and we selected pooled antisense IL-8 transfected PC3-LN4 cells, PC3M-LN4(AS IL-8), the highest IL-8 expressing clone, PC3M-LN4(AS IL-8 High), and the lowest IL-8 expressing clone, PC3M-LN4(AS IL-8 Low), according to the expression level of IL-8 mRNA and protein as determined by northern blot analysis and ELISA, respectively.

Northern Blot Analysis. Polyadenylated mRNA was extracted directly from the tumors or from 10^8 cultured cells using the Fasttrack™ mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA was electrophoresed on to 1% denatured formaldehyde agarose gel, electrotransferred to Genescreen nylon membrane (DuPont Co., Boston, MA), and crosslinked with a UV Stratalinker 1800 (Strategene, LA Jolla, CA) at 120,000 mJ/cm^2. Filters were washed twice at 65°C with 30 mM NaCl/3 mM sodium citrate-0.1% sodium dodecyl sulfate (wt/vol). The membranes were then hybridized and probed for IL-8, bFGF, VEGF, and MMP-9; the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to control for loading. The cDNA probes used were (1) a 0.5-kb *Eco*RI cDNA fragment corresponding to human IL-8 (a gift of Dr. K. Matsushima, Kanazawa, Japan),[23] (2) a 1.4-kb cDNA fragment of bovine bFGF,[33] (3) a 204-kb fragment of human VEGF cDNA inserted in a pGEM-based construct (a gift Dr. B. Berse, Harvard Medical School, Boston, MA),[34] (4) a 1.0-kb kbN-I cDNA fragment corresponding to human MMP-9,[21 and]

(5) a 1.28-kb fragment from pR GAPDH cut with *Pst*I.[35] The insert was excised with *Bam*HI and *Eco*RI. Each cDNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (BIO 101, Inc., La Jolla, CA), and radiolabeled by a random primer technique using a commercial kit (Boehringer Mannheim Corp., Indianapolis, IN) and α-^{32}P-deoxycytidine triphosphate (Amersham Corp., Arlington Heights, IL)[36]. The steady-state expression of IL-8, bFGF, VEGF, and MMP-9 mRNA transcripts was quantified by densitometry of autoradiographs using the Image Quant software program (Molecular Dynamics, Sunnyvale, CA); each sample measurement was calculated as the ratio of the average areas of the specific mRNA transcripts to the 1.3-kb GAPDH mRNA transcript in the linear range of the film.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-8, bFGF, and VEGF. Viable cells (5×10^3) were seeded in a 96-well plate. Conditioned medium was removed after 24 hours, and the cells were washed with 200 µl of Hank's balanced salt solution (HBSS), and 200 µl of 10% bovine serum supplemented by fresh MEM was added. Twenty-four hours later, IL-8 and VEGF in cell-free culture supernatants and cell-associated bFGF in freeze-thaw cell lysates were determined using the commercial Quantine ELISA kit (R&D System, Minneapolis, MN). The protein concentration for each factor was then determined by optical density comparison to the standard curve. Results were expressed as numbers of cells[37].

Growth Curve. Viable cells (1×10^3) were seeded in a 96-well plate. Conditioned medium was removed after 24 hours, and the cells were washed with 200 µl of HBSS. Either 200 µl of fresh CRPMI medium or CMEM/G418 conditioned medium added. Every 24 hours, the numbers of viable cells in each cell line were determined by optical density comparison. The doubling time of each cell line was determined by plotting the optical density on a semilogarithmic axis versus time (Cricket Software, Malvern, PA). The doubling times of the PC3-P sense IL-8 transfectants (IL-8: 40.1 hours, IL-8 Low: 38.1 hours, IL-8 High: 41.2 hours) were similar to those of PC3-P: 38.6 hours; and PC3-P(Neo)(39.1 hours), and the doubling time of the PC3M-LN4 antisense IL-8 transfectants (AS IL-8: 22.5 hours, AS IL-8 Low: 21.5 hours, AS IL-8 High: 21.7 hours) was similar to those of PC3M-LN4 (22.0 hours) and PC3M-LN4(Neo) (22.6 hours).

Collagenase Activity. To determine collagenase activity, electrophoresis of serum-free conditioned medium was performed as described previously[38]. Cells (5×10^3) were seeded in six-well plates and grown to 60% to 70% confluence. The cells were washed with HBSS and grown for 24 hours in serum-free medium, and the collagenase activity of the supernatant fluid was determined. Identification of a transparent band at 72 or 92 kDa on the Coomasie blue background of the slab gel was considered positive to indicate enzymatic activity.

To determine whether the increase in MMP-9 activity is mediated by IL-8, we incubated parental PC3-P cells in the presence of different doses (0-20μg/ml) of human recombinant IL-8 (rIL-8), and the activity of MMP-9 was determined. We then determined the increased activity of MMP-9 by rIL-8 was inhibited by neutralization by using an anti-IL-8 antibody (100 μg/ml), with a non-specific IgG (100 μg/ml) as a control.

PCR Analysis. RT-PCR analysis was performed, as previously described[39]. Briefly, total cellular RNA (1 mg) extracted from various cell lines was transcribed into cDNA using downstreaming primers of high affinity type and low affinity type IL-8 receptors, respectively (Reverse Transcription System, Promega). The RT reaction was performed at 42°C for 50 min. PCR was performed with 40 cycles of denaturation(94°C for 1.5 min), annealing (58°C for 45 sec), and extension (72°C for 2.5 min) and 7 min of extension after completion of all cycles. Amplified fragments were analyzed on the 2% gel and bands of expected sizes were confirmed by sequencing. The primer sequences used were as follows: for low affinity IL-8 receptor, sense 5–AGT-TCT-TGG-CAC-GTC-ATC-G- 3'and antisense 5'–CTT-GGA-GGT-ACC-TCA-ACA-GC- 3' for IL-8 receptor type A and sense 5' – ACA-TTC-CTG-TGC-AAG-GTG-G- 3' and antisense 5' – CAG-GGT-GAA-TCC-GTA-GCA-GA - 3' for IL-8 receptor type B.

Invasion Assay through Matrigel. Polyinylprolidone-free polycarbonate filters (8 μm pore size; Nuclepore; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with a mixture of basement membrane components (Matrigel, 25 μg/filter) and placed in modified Boyden chambers. The cells (2×10^5) were released from their culture dishes by short exposure to EDTA (1 mmol/L), centrifuged, resuspended in 0.1% bovine serum albumin-Dulbecco's minimal essential medium, and placed in the upper compartment of the Boyden chamber. Fibroblast-conditioned medium in the

lower compartment served as a chemoattractant. After incubation for 6 hours at 37°C, the cells on the lower surface of the filter were stained with Diff-Quick (American Scientific Products, McGaw Park, IL) and quantified with a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corporation, Tokyo, Japan). The results were expressed as the average number of cells in the five highest spots identified within a single 200× field on the lower surface of the filter[40].

Animals. Male athymic BALB/c nude mice were obtained from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a laminar-airflow cabinet under pathogen-free conditions and used at 8 to 12 weeks of age. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U. S. Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health.

Orthotopic Implantation of Tumor Cells. Cultured PC3-P, PC3M-LN4, Neo, sense and antisense IL-8 transfected cells (60% to 70% confluent) were prepared for injection as previously described[32,41]. Mice were anesthetized with methoxyflurane. For orthotopic implantation, a lower midline incision was made, and viable tumor cells (2×10^6/40 μL) HBSS were implanted into the dorsal prostate lobes using a 30-gauge needle with a 1-ml disposable syringe and a calibrated push button-controlled dispensing device (Hamilton Syringe Company, Reno, NV). Formation of a bulla indicated a satisfactory injection. The prostate was returned to the abdominal cavity and the abdominal wall was closed with a single layer of metal clips. Mice were killed 6 weeks after implantation of tumor cells. The primary tumors were removed and weighed, and the presence of metastases (in the lymph nodes) was determined grossly and microscopically. The prostates were then either quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, placed in OCT compound (Miles Laboratories, Elkhart, IN), or mechanically dissociated and put into tissue culture.

***In Situ* mRNA Hybridization (ISH) Analysis.** Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the

cDNA sequence: IL-8 (CTC'CAC'AAC'CCT'CTG'CAC'CC), 66% guanosine cytosine (GC) content[23]; bFGF(CGG'GAA'GGC'GCC'GCT'GCC'GCC'), 85.7% GC content[33]; VEGF /VPF(TGG'TGA'TGT'TGG'ACT'CTT'CAG'TGG'GCU), 57.7% GC content[34]; MMP-9 (CCG'GTC'CAC'CTC'GCT'GGC'GCT'-CCG'GU), 80.0% GC content[21]. The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm; these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by northern blot analysis.[41] A poly $d(T)_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling, with the use of standard phosphoramidite chemical methods (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted to a stock solution at 1 μg/μL in 10 mmol/L Tris (pH 7.6) and 1 mmol/L EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics, Huntsville, AL).

In situ mRNA hybridization was performed as described previously with minor modifications,[43,44] using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA)[45]. Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific)[43,44]. The slides were placed in the Microprobe slide holder, dewaxed, and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 minutes at 45°C, and the samples were then washed three times with 2×SSC for 2 minutes at 45°C. The samples were incubated with alkaline phosphatase-labeled avidin for 30 minutes at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 minute, and incubated with a chromogen substrate for 15 minutes at 45°C. Additional incubation with fresh chromogen substrate was performed if necessary to enhance a weak reaction in this assay, a red staining indicated a positive reaction. Control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of Chromagen alone.

Quantification of Color Reaction. Stained sections were examined in a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip, charge-coupled device

color camera (model DXC-969 MD; Sony Corp., Tokyo, Japan). The images were analyzed using the Optimas image analysis software (version 4.10, Bioscan Bothell, WA). The slides were prescreened by one of the investigators to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue mode of the color camera. All subsequent images were quantified based on this threshold. The integrated optical density of each field was determined based on its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained, so the optical density was due solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity of staining was determined by comparison with the integrated optical density of poly $d(T)_{20}$. The results were presented as the number of cells for each cell line compared with the control, which was set to 100[37].

Immunohistochemistry (IHC). For immunohistochemical analysis, frozen tissue sections (8 μm thick) were fixed with cold acetone. Tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with PBS, and antigen retrieval was performed with pepsin for 12 minutes, and endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 minutes. The samples were washed three times with PBS and incubated for 20 minutes at room temperature with a protein-blocking solution of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 hours at 4°C with the appropriate dilution (1:100) of rat monoclonal anti-CD31 antibody (Pharmingen, San Diego, CA),[46] a 1:50 dilution of a rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:500 dilution of rabbit polyclonal anti-bFGF antibody (Sigma Chemical Co., St. Louis, MO), a 1:500 dilution of rabbit polyclonal anti-VEGF/VPF antibody (Santa Cruz Biotech, Santa Cruz, CA), or a 1:100 dilution of mouse monoclonal anti-MMP-9 antibody (Oncogene Research Products, Cambridge, MA). The samples were then rinsed four times with PBS and incubated for 60 minutes at room temperature with the appropriate dilution of the secondary antibody:peroxidase-conjugated anti-rat immunoglobulin G (IgG) (H+L) (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA), anti-rabbit IgG, $F[ab]_2$ fragment (Jackson ImmunoResearch Laboratory, Inc.) or anti-mouse IgG_1 (Pharmingen, San Diego, CA). The slides

were rinsed with PBS and incubated for 5 minutes with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill's hematoxylin (Biogenex Laboratories, San Ramon, CA), and again washed three times with PBS. The slides were mounted with Universal Mount mounting medium (Research Genetics).

Quantification of Microvessel Density. Microvessel density was determined by light microscopy after immunostaining frozen sections with anti-CD31 antibodies as described by Weidner et al.[47] Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The tissue was recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corporation). The density of microvessels was expressed as the average number of the five highest area identified within a single 200×field.

Quantification of Intensity of Immunostaining. The intensity of immunostaining of IL-8, bFGF, VEGF, and MMP-9 was quantitated in each sample by an image analyzer using the Optimas software program (Bioscan). Three different areas in each sample were quantified to yield an average measurement. The results were presented as the number of cells for each cell line compared with the control which was set to 100[37].

MMP-9 mRNA Half-Life Studies. To determine the effect of IL-8 on MMP-9 mRNA stability, PC3-P, PC3-P(Neo), and PC3-P(IL-8) cells and PC3M-LN4, PC3M-LN4(Neo), and PC3M-LN4(AS IL-8) cells were incubated for 24 hours. Further transcription in the cells was then blocked by the addition of ActD (Calbiochem-Novabiotechnology, Inc., Lake Placid NY; final concentration of 5 μg/ml). Total RNA was extracted from the cells at 0, 1, 2, and 4 hours after the addition of ActD, and MMP-9 mRNA expression was determined by northern blot analysis. MMP-9 mRNA expression of each time point was compared with the control value (total RNA extracted from cells prior to ActD treatment was arbitrarily defined as 100%). The half-life of MMP-9 mRNA

was determined by plotting relative MMP-9 mRNA expression levels on a semilogarithmic axis versus time (Cricket Software).

CAT Assay. Using the FuGENE 6 protocol (Boehringer Mannheim Corp.), we transfected with the basic CAT expression vector with no promoter/enhancer sequences (pCAT-basic) or a control plasmid with SV40 promoter and enhancer (pCAT-control; Promega, Madison, WI) into PC3-P cells, sense transfected PC3-P cells, PC3M-LN4 cells, antisense-transfected PC3-LN4 cells, and each Neo transfectant. One copy of the human MMP-9 promoter region spanning nucleotides -390 to +290 was ligated upstream of the basic CAT expression vector. We transfected 5×10^3 cells/well in a six-well tissue culture dish with 2.5 μg of the reporter CAT constructs and 2.5 μg of a β-actin expression plasmid. After 48 hours, extracts were prepared from all plates, normalized for β-actin activity, and assayed for CAT activity[48] as Hudson *et al.*[49] described previously. The CAT assay was quantified by densitometry of autoradiographs with the use of the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA) and was evaluated as the ratio of acetylated species to all species.

Statistical Analysis. The statistical differences in vessel counts and staining intensity for IL-8, bFGF, VEGF, and MMP-9 of prostate tumors were analyzed by the Mann-Whitney U-test. The incidences of tumor and metastasis were statistically analyzed by chi-squared test. A value of $p < 0.05$ was considered significant.

Results

In Vitro Expression of IL-8, bFGF, VEGF, and MMP-9. Northern blot analysis for IL-8, bFGF, and VEGF steady-state gene expression by PC3-P, PC3-P(IL-8), PC3-P(IL-8 Low), PC3-P(IL-8 High) and PC3-P(Neo) is shown in Figure 1A., and that by PC3M-LN4, PC3M-LN4(AS IL-8), PC3M-LN4(AS IL-8 Low), PC3M-LN4(AS IL-8 High) and PC3M-LN4(Neo) is shown in Figure 1B. The level of expression is shown as the ratio of mRNA expression by the transfectants to that by the corresponding parental and Neo transfectant cell lines (which in both cases were equivalent for all three factors). IL-8 mRNA expression levels were increased 10.4- and 15.1-fold higher in PC3-P(IL-8) and PC3-P(IL-8 High), respectively than in either PC3-P or PC3-P(Neo), while there was no change in the mRNA expression of bFGF or VEGF. The mRNA expression levels of IL-8 in PC3M-LN4(AS IL-8) and in PC3M-LN4(AS IL-8 Low) were only about one fifth and one tenth of those for either either PC3M-LN4 or PC3M-LN4(Neo), respectively, while there was no change in the mRNA expression of bFGF or VEGF. IL-8, bFGF, and VEGF protein production by PC3-P, PC3M-LN4, and the transfected cell lines was evaluated by ELISA (Figure 2.). Changes in protein expression by the transfectants paralleled the changes seen in mRNA expression. IL-8 expression levels were 3.0-, and 4.0-fold higher in the PC3-P(IL-8) and PC3-P(IL-8 High) cells, respectively, than in the PC3-P parental cell line. PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) cell lines were only one quarter and one tenth, of those in the parental PC3M-LN4 cells, respectively. Basic FGF and VEGF protein expression levels were unchanged in all cell lines following transfection.

Metalloproteinase Expression Following IL-8 Transfection. Since IL-8 regulates protease activity by human melanoma, we evaluated whether MMP-9 expression was altered in the PC3-P and PC3M-LN4 cells by transfection with sense or antisense IL-8 transcripts. Figure 1. shows that MMP-9 mRNA expression levels were 3.0- and 6.0-fold higher in PC3-P(IL-8) and PC3-P(IL-8 High) cells, respectively, compared with their controls, but the values for both PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low), cells were only one third of that of their controls. These results demonstrate that IL-8 regulates MMP-9 mRNA expression by the PC3-P and PC3M-

LN4 human prostate cancer cells. Our results are consistent with reports that IL-8 regulates MMP expression by malignant melanoma. [30, 31]

Collagenase Activity. To demonstrate that MMP-9 expressed by the transfected cells is biologically active, collagenase activity of the transfected cells was determined by zymography (Figure 3.). By densitometry, the collagenase activity of PC3-P(IL-8) and PC3-P(IL-8 High) was increased 6.0- and 7.0-fold, compared with either PC3-P or PC3-P(Neo)(Figure 3A.), respectively, while that of PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) was decreased 2.5- and 5.0-fold compared with either PC3M-LN4 or PC3M-LN4(Neo), respectively, (Figure 3B.).

We next analyzed whether the increase in MMP-9 activity is mediated by IL-8 (Fig. 4). To that end, parental PC3-P cells were incubated in the presence of different doses of human recombinant IL-8 (rIL-8), and the activity of MMP-9 was determined. The results shown in Figure 4A. indicate that IL-8 caused an increase in the activity of MMP-9 in a dose-dependent manner. Moreover, the increased activity of MMP-9 by rIL-8 was inhibited by neutralization with anti-IL-8 antibody (Figure 4B.).

RT-PCR Analysis. RT-PCR analysis revealed that PC3-P, PC3-P(Neo) and sense-IL-8 transfectants (IL-8, IL-8 Low, and IL-8 High) (Figure 5A), as well as PC3M-LN4, PC3M-LN4(Neo) and antisense-IL-8 transfectants (AS IL-8, AS IL-8 Low, and AS IL-8 High) (Figure 5B) express mRNA for both type IL-8 receptors.

Invasion Assay through Matrigel. We next analyzed whether the expression of MMP-9 and collagenase activity by the IL-8-transfected cells correlated with invasion through the basement membrane. PC3-P(IL-8) and PC3-P(IL-8 High) cells exhibited increased invasion through Matrigel-coated filters, with 3.0- and 4.0-fold increases, compared with either PC3-P or PC3-P(Neo)($P < 0.005$), respectively (Figure 6A.). Invasion by PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) was 80% and 85% lower, compared with invasion by PC3M-LN4 or PC3M-LN4(Neo)($P < 0.005$), respectively (Figure 6B.).

CAT Activity. The MMP-9 promoter (-390 to +290) was linked upstream of the CAT reporter gene and transfected into PC3-P, PC3-LN4, sense IL-8 transfected, antisense IL-8 transfected, and the Neo-transfected cells to examine the effect of IL-8 expression on MMP-9 transcription. Forty-eight hours after transfection, cell extracts were prepared, and equivalent amounts of extracts exhibiting the same β-actin activity were tested for CAT activity. CAT activity driven by MMP-9 promoter in PC3-P(IL-8) and PC3-P(IL-8 High) was increased 1.5- and 2.9-fold (Figure 7A.) compared with either PC3-P or PC3-P(Neo), respectively, and decreased 2.5- and 10.0-fold by PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) (Figure 7B.) compared with either PC3M-LN4 or PC3M-LN4(Neo), respectively. CAT activity driven by the SV-40 promoter was the same in both cell populations and served as an additional internal control for transfection efficiency.

To determine the mechanism by which IL-8 enhanced the expression of MMP-9 mRNA, the stability of MMP-9 mRNA was investigated by examining its half-life. The half-life of MMP-9 mRNA of PC3-P(IL-8) was similar to that of PC3-P or PC3-P(Neo) and that of PC3-LN4(AS IL-8) was similar to that of PC3M-LN4 or PC3M-LN4(AS Neo) (data not shown).

Tumorigenicity and Production of Metastasis. To evaluate whether IL-8 expression regulates tumorigenicity and metastasis of androgen-independent prostate cancer, we implanted PC3-P, PC3M-LN4, Neo- transfected and the IL-8 sense and antisense-transfected cells into the prostate of athymic nude mice and evaluated tumor growth and metastasis 6 weeks later (Table 1.). The PC3-P(IL-8) and PC3-P(IL-8 High) tumors were larger than the PC3-P and PC3-P(Neo) tumors (mean weight [range], 1,270 mg: [258-1,850 mg] ($P < 0.005$), 1,975 mg [355-2,915 mg] ($P < 0.005$) vs. 55 mg [24-480 mg], 120 mg [27-443 mg]). Moreover, the incidences of spontaneous lymph node metastasis, as well as tumor burden within the metastatic lymph nodes, were significantly greater for the PC3-P(IL-8) and PC3-P(IL-8 High) cell lines than for the PC3-P and PC3-P(Neo) (incidences: 8 of 8, 9 of 9, 4 of 7, 4 of 9, respectively ($P < 0.05$); mean lymph node weight [range], 93 mg [36-124 mg], 131 mg [55-188 mg], 20 mg [16-28 mg], 24 mg [15-32 mg], respectively) ($P < 0.005$). Conversely, the tumorigenicity of PC3M-LN4 was significantly inhibited when IL-8 expression was reduced by antisense IL-8 transfection. Only 2 of 8 mice implanted with PC3M-LN4(AS IL-8) (tumor weights 33 and 36 mg) and PC3M-LN4(AS IL-8 Low) (tumor

weights 31 and 39 mg) developed tumors at 6 weeks ($P < 0.01$) compared with the mice implanted with PC3M-LN4 and PC3M-LN4(Neo). These tumors were not apparent histologically but grew out as tissue explants in culture. Tumorigenicity was also inhibited in PC3M-LN4(AS IL-8 High), but this difference did not reach statistical significance. There was a significant reduction in spontaneous lymph node metastases at 6 weeks in mice implanted with PC3M-LN4(AS IL-8), PC3M-LN4(AS IL-8 Low), or PC3M-LN4(AS IL-8 High) (no mice developed metastasis) compared with mice implanted either PC3M-LN4 or PC3M-LN4(Neo) (all mice developed metastasis) ($P < 0.0005$). Therefore, IL-8 expression by PC3-P and PC3M-LN4 regulates both tumorigenicity and metastasis in androgen-independent prostate cancer.

***In Vivo* Expression of IL-8, bFGF, VEGF, and MMP-9.** IL-8, bFGF, VEGF, and MMP-9 mRNA and protein were evaluated by ISH (Table 2., Figure 8.) and IHC (Table 2., Figure 9.), respectively. The mRNA and protein expressions of IL-8 and MMP-9 were increased 2.5- and 3.0-fold in the PC3-P(IL-8) and PC3-P(IL-8 High) tumors, respectively, relative to either PC3-P or PC3-P(Neo). The PC3M-LN4(AS IL-8 High) tumors showed a 42% reduction in the mRNA and protein expression of IL-8 and a 33% reduction in the mRNA and protein expression of MMP-9 relative to either PC3M-LN4 or PC3M-LN4(Neo) tumors. There was no change in the mRNA and protein expression of bFGF or VEGF in the IL-8-transfected tumors.

Tumor Angiogenesis. Tumor-induced neovascularization (as indicated by MVD) was determined by IHC using anti-CD31 antibodies (Table 2., Figure 9.). The numbers of $CD31^+$ microvessels counted per 200× field were 40 ± 9 and 45 ± 6 in PC3-P and PC3-P(Neo), respectively, compared with 80 ± 13 and 91 ± 18 in PC3-P(IL-8) and PC3-P(IL-8 High), respectively ($P < 0.005$). Conversely, antisense IL-8 transfection of PC3M-LN4 significantly decreased MVD from 100 ± 20 and 104 ± 23 in the PC3M-LN4 and PC3M-LN4(Neo) tumors, respectively, to 47 ± 14 in the PC3M-LN4(AS IL-8 High) tumors ($P < 0.005$) (Table 2., Figure 9.). Since the PC3M-LN4(IL-8) and PC3M-LN4(AS IL-8 Low) cells grew only as explants tissue culture, we could not evaluate MVD. These studies indicate that tumor-induced neovascularization correlates directly with IL-8 expression, tumorigenicity, and metastasis.

Discussion

Prostate cancer growth and metastasis depend upon the ability of the cancer to induce its own blood supply.[18,21] This process of angiogenesis depends on the outcome between stimulatory and inhibitory regulation by the tumor and its microenvironment[14-16]. Human prostate cancer expresses a number of angiogenesis factors including VEGF,[17,18] bFGF,[19,20] and IL-8.[18,21] The metastatic potential of the LNCaP prostate cancer cell line correlates with VEGF expression[50], and that of the PC3 line with bFGF and IL-8,[19,21] but there has been no direct evidence that these factors are involved in the growth and metastasis of prostate cancer. Luca et al.[31] enforced expression of IL-8 in the SB-2 melanoma cell line by sense transfection and demonstrated that IL-8 regulated tumorigenicity and metastasis in human melanoma. The purpose of our study was to provide direct evidence for the role of IL-8 in regulating the tumor induced neovascularization and subsequent tumor growth and metastasis of human prostate cancer growing within the prostate of athymic nude mice.

We enforced IL-8 expression by transfecting the poorly tumorigenic and poorly metastatic human prostate cancer cell line PC3-P (which expresses relatively low levels of IL-8) with the sense IL-8 construct and were able to establish several cell lines that overexpress IL-8 relative to the original PC3-P cell line. These sense IL-8 transfected cells demonstrated enhanced tumorigenicity and metastasis compared with the PC3-P or PC3-P(Neo) cells, and demonstrated enhanced tumor-induced neovascularization, growth within the prostate, and spontaneous metastasis to the lymph nodes. Conversely, following antisense IL-8 transfection we were able to reduce IL-8 expression by the highly tumorigenic and metastatic PC3M-LN4 cell line (which express relatively high levels of IL-8) and to inhibit tumor-induced neovascularization, growth within the prostate, and metastasis. Since neither bFGF nor VEGF expression was altered by IL-8 transfection, we conclude that these effects are independent of the activity of these angiogenesis factors. Since IL-8 tranfection did not affect in-vitro proliferation of PC3-P or PC3M-LN4, the effects on growth and metastasis are independent of proliferation, although the cells do have both type A (CXCR1) [51,52] and type B (CXCR2) [51,52] of the IL-8 receptors. Therefore, our results provide direct evidence for the involvement of IL-8 in the induction of in vivo angiogenesis and in the subsequent growth and metastasis of prostate cancer. These results are similar to previous reports in which transfection with

VEGF or bFGF increased MVD and enhanced tumor growth and metastasis of melanoma and breast cancer[53-55].

The metastatic potential of prostate cancer depends upon the expression of several metastasis related genes such as IL-8, that regulate endothelial cell proliferation and capillary morphogenesis,[27] and other genes, such as MMP-9, that regulate the degradation of the extracellular matrix[56,57]. The local production of MMPs or other proteases such as plasminogen activator by prostate cancer cells or stroma facilitates the local degradation of the ECM and results in tumor invasion and subsequent metastasis[56-59]. The proteolytic effect of MMPs facilitates the migration of endothelial cells through the altered ECM toward the source of the angiogenic stimulus; in this manner, MMPs are an integral component of the angiogenesis pathway. The highly metastatic PC3M-LN4 express high levels of MMP-9 compared with the poorly metastatic PC3-P cell line. Recently, Luca et al.[31] reported that IL-8 regulates MMP-2 activity by malignant melanoma cells.[31] They transfect the melanoma cell line SB-2 with the sense IL-8 transcript and upregulated MMP-2 expression and collagenase activity, and they considered this upregulation of collagenase activity to be an important mechanism that explained the associated increase in metastatic ability demonstrated by the sense-transfected SB-2 cells. Similarly, we found that the activity of both MMP-9 by human prostate cancer cells directly correlated with their expression of IL-8. Moreover, when we altered the expression of IL-8 by sense or antisense transfection, we observed a corresponding change in MMP expression and activity both in vitro and in vivo. The MMP induced by sense transfection was biologically active, since it increased collagenase activity and increased cellular invasion through matrigel. When MMP activity was reduced following antisense transfection both collagenase activity and invasion through matrigel decreased. The altered local growth of the antisense- transfected tumors may reflect a relative growth inhibition secondary to the inability to induce a robust microcirculation, and the loss of metastatic potential may be due to both a decrease in the tumor-induced neovascularization by IL-8 and a reduction in invasion due to the reduction in MMP-9 activity. Conversely, increased IL-8 expression by the sense IL-8-transfected prostate cancer cells may explain their enhanced tumorigenicity, while both increased IL-8 and MMP expression may contribute to their increased metastatic potential.

MMP expression by IL-8 is probably regulated at the level of transcription. We evaluated MMP-9 mRNA stability and the level of gene transcription of MMP-9 in IL-8 transfectants and control cells. Although the expression of MMP-9 mRNA varied among the IL-8 transfectants and

controls, the stability of MMP-9 mRNA was not changed by transfection with sense or antisense IL-8. However, CAT activity driven by the MMP-9 promoter was upregulated in IL-8 sense transfectants and downregulated after antisense transfection. Basic FGF regulates MMP-9 expression by human bladder cancer. Since bFGF levels were not affected by IL-8 transfection, the regulation of MMP-9 transcription in PC3-P andPC3M-LN4 cells is independent of bFGF and likely regulated by IL-8. These results are in keeping with the report of Luca et al.[31] who found that IL-8 regulated MMP-2 gene transcription.

In summary, our present study demonstrates that IL-8 regulates angiogenesis, tumorigenesis, and metastasis by androgen-independent human prostate cancer. This effect is mediated in part by the regulation of the expression and activity of MMP-9.

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Figure Legends

Figure 1. Northern blot analysis of mRNA for IL-8, bFGF, VEGF, and MMP-9 in the poorly tumorigenic and poorly metastatic human prostate cancer cell line PC3-P, Neo transfectant PC3-P(Neo) and sense IL-8 transfectants PC3-P(IL-8), PC3-P(IL-8 Low), PC3-P(IL-8 High) (A) and in the highly metastatic human prostate cancer cell line PC3M-LN4, Neo transfectant PC3M-LN4(Neo) and antisense IL-8 transfectants PC3M-LN4(AS IL-8), PC3M-LN4(AS IL-8 Low), PC3M-LN4(AS IL-8 High) (B). Difference in expression is shown by the ratio of mRNA expression of transfectants to that of parental cells (defined as 1.0). GAPDH served as control for loading. IL-8 expressions were increased 10.4- and 15.1-fold in the PC3-P(IL-8) and PC3-P(IL-8 High) lines, respectively, while there was no change in the mRNA expression of bFGF or VEGF. mRNA expression of IL-8 by PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) were decreased 5.0- to 10.0-fold, respectively, while there was no change in the mRNA expression of bFGF or VEGF. MMP-9 mRNA expressions were increased 3.0- and 6.0-fold in PC3-P(IL-8) and PC3-P(IL-8 High) cells respectively, and reduced 3.3- and 3.3-fold by PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low), respectively, following transfection with IL-8 sense or antisense transcripts.

Figure 2. Protein expression of IL-8, bFGF, and VEGF in PC3-P, Neo transfectant PC3-P(Neo) and sense IL-8 transfectants PC3-P(IL-8), PC3-P(IL-8 Low), PC3-P(IL-8 High) (A) and in PC3M-LN4, Neo transfectant PC3M-LN4(Neo) and antisense IL-8 transfectants PC3M-LN4(AS IL-8), PC3M-LN4(AS IL-8 Low), PC3M-LN4(AS IL-8 High) (B) was analyzed by ELISA. Cells (5×10^3/well) were cultured for 48 hours in RPMI or CMEM/G418. Cell-free culture supernatant was analyzed for IL-8 and VEGF. Cell-lysate was analyzed for bFGF. IL-8 expression was increased 3.0-, and 4.0-fold by the PC3-P(IL-8) and PC3-P(IL-8 High) cells, respectively, and decreased 4.0- to 10.0-fold by PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low), respectively. Changes in protein expression by the transfectant paralleled the changes seen in mRNA expression. Basic FGF and VEGF protein expressions were unchanged.

Figure 3. Gelatinolytic activity of conditioned medium of PC3-P, Neo transfectant PC3-P(Neo) and sense IL-8 transfectants PC3-P(IL-8), PC3-P(IL-8 Low), PC3-P(IL-8 High) (A) and of PC3M-LN4, Neo transfectant PC3M-LN4(Neo) and antisense IL-8 transfectants PC3M-LN4(AS IL-8),

PC3M-LN4(AS IL-8 Low), PC3M-LN4(AS IL-8 High) (B). CMEM was used as internal control. Difference in expression is expressed as the ratio of gelatinolytic activity of transfectants to that of parental cells (defined as 1.0). The collagenase activity of PC3-P(IL-8) and PC3-P(IL-8 High) cells was increased 6.0- and 7.0-fold, respectively, and that of PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) cells was decreased 2.5- to 5.0-fold.

Figure 4. Regulation of MMP-9 activity by IL-8. We next analyzed whether the increase in MMP-9 activity is mediated by IL-8. Parental 253J-P cells were incubated in the presence of different doses of human recombinant IL-8 (rIL-8) (0-20 μg/ml), and the activity of MMP-9 was determined. The results shown in Figure 4A. indicate that IL-8 caused an increase in the activity of MMP-9 in a dose-dependent manner. Moreover, the increased activity of MMP-9 by rIL-8 was inhibited by neutralization with anti-IL-8 antibody(100 μg/ml)(B).

Figure 5. RT-PCR analysis was performed by primer sequences used were sense 5' – AGT-TCT-TGG-CAC-GTC-ATC-G- 3'and antisense 5' – CTT-GGA-GGT-ACC-TCA-ACA-GC- 3' for IL-8 receptor type A and sense 5' – ACA-TTC-CTG-TGC-AAG-GTG-G- 3' and antisense 5' – CAG-GGT-GAA-TCC-GTA-GCA-GA - 3' for IL-8 receptor type B.RT-PCR analysis revealed that PC3-P, PC3-P(Neo) and sense-IL-8 transfectants (IL-8, IL-8 Low, and IL-8 High) (5A),as well as PC3M-LN4, PC3M-LN4(Neo) and antisense-IL-8 transfectants (AS IL-8, AS IL-8 Low, and AS IL-8 High) (5B) express mRNA for both type IL-8 receptors.

Figure 6. Migration of cells (2×10^5) of sense transfectants (A) and antisense transfectants (B) through Matrigel-coated filters into fibroblast-conditioned medium as a source of chemoattractants, expressed as the average number of cells in the five highest spots identified within a single 200× field on the lower surface of the filter. The number of migrated cells that penetrated through the Matrigel-coated filters increased 3.0- and 4.0-fold for the PC3-P(IL-8) and PC3-P(IL-8 High) cells, respectively ($P < 0.005$), while the numbers of cells migrating were markedly reduced in PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) cells relative to controls, with 80%, and 85% reductions respectively, ($P < 0.005$).

Figure 7. MMP-9 mRNA half-life studies. PC3-P, PC3-P(Neo), PC3-P(IL-8), and PC3M-LN4, PC3M-LN4(Neo), PC3M-LN4(AS IL-8) cells were incubated for 24 hrs prior to exposure to ActD (5μg/ml). Total RNA was extracted from the cells at 0, 1, 2, 4 hrs after the addition of ActD, and Northern blot analysis was performed for MMP-9 mRNA expression. Relative MMP-9 mRNA expression was calculated, and the half-life was determined by plotting representative relative MMP-9 expression values on a semilogarithmic scale. The half-life of MMP-9 mRNA in PC3-P(IL-8) and PC3M-LN4(AS IL-8) cells was similar to that in PC3-P or PC3-P(Neo), and PC3M-LN4 or PC3M-LN4(Neo), respectively.

Figure 8. Effect of IL-8 expression on CAT activity driven by the MMP-9 promoter in sense (A) and antisense (B) IL-8 transfectants. The CAT activity was evaluated as the ratio of acetylated species to all species. Differences in expression are shown as the ratio of CAT activity of transfectants to that of parental cells (defined as 1.0). CAT activity levels driven by MMP-9 promoter in PC3-P(IL-8) and PC3-P(IL-8 High) cells were increased 1.5-, and 2.9-fold, respectively, compared with either PC3-P or PC3-P(Neo) and decreased 2.5- and 10.0-fold by PC3M-LN4(AS IL-8) and PC3M-LN4(AS IL-8 Low) cells, respectively, compared with either PC3M-LN4 or PC3M-LN4(Neo).

Figure 9. ISH in PC3-P, Neo transfectant PC3-P(Neo) and sense IL-8 transfectants PC3-P(Neo) and sense IL-8 transfectants PC3-P(IL-8), PC3-P(IL-8 Low), PC3-P(IL-8 High) (A), and in PC3M-LN4, Neo transfectant PC3M-LN4(Neo) and antisense IL-8 transfectants PC3M-LN4(AS IL-8), PC3M-LN4(AS IL-8 Low), PC3M-LN4(AS IL-8 High) (B). The intensity of staining was determined by comparison with the integrated optical density of poly $d(T)_{20}$, which was set to 100.

The mRNA expression of IL-8 and MMP-9 was increased 2.5- and 3.0-fold in the tumor of PC3-P(IL-8) and PC3-P(IL-8 High) relative to PC3-P or PC3-P(Neo), respectively. The tumor of PC3M-LN4(AS IL-8 High) showed a 40% reduction in the mRNA expression of IL-8 and a 30% reduction in the mRNA expression of MMP-9 relative to that of either parental PC3M-LN4 or PC3M-LN4(Neo).

Figure 10. Immunohistochemistry and microvessel density in PC3-P, Neo transfectant PC3-P(Neo), sense IL-8 transfectants PC3-P(Neo) and sense IL-8 transfectants PC3-P(IL-8), PC3-P(IL-

8 Low), and PC3-P(IL-8 High) (A) and in PC3M-LN4, Neo transfectant PC3M-LN4(Neo) and antisense IL-8 transfectants PC3M-LN4(AS IL-8), PC3M-LN4(AS IL-8 Low), and PC3M-LN4(AS IL-8 High) (B). Three different areas in each sample were quantified to obtain an average measurement of intensity of immunostaining. The density of microvessels was expressed as the average of the five highest area identified within a single 200× field. The protein expressions of IL-8 and MMP-9 were increased 2.5- and 3.0-fold in the tumor of PC3-P(IL-8) relative to that of either parental PC3-P or PC3-P(Neo), respectively. The tumor of PC3M-LN4(AS IL-8 High) showed a 40% reduction in the protein expression of IL-8 and a 30% reduction in the protein expression of MMP-9 relative to that of either PC3M-LN4 or PC3M-LN4(Neo). The number of $CD31^+$ microvessels counted per 200× field in prostate tumors of PC3-P(IL-8) cells was increased from 40 ± 9 or 45 ± 6 in the tumors of parental PC3-P or PC3-P(Neo) to 80 ± 13 and 91 ± 18 in that of PC3-P(IL-8) and PC3-P(IL-8 High) cells, respectively ($P < 0.005$). The number of $CD31^+$ microvessels was reduced from 100 ± 20 or 104 ± 23 in the tumors of parental PC3M-LN4 or PC3M-LN4(Neo), respectively, to 47 ± 14 in that of PC3M-LN4(AS IL-8 High) cells ($P < 0.005$).

Table 1 Tumorigenicity and Production of Spontaneous Metastases after Orthotopic Implantation of PC3-P, PC3-P (Neo), and sense IL-8 Transfectants and of PC3M-LN4,PCM-LN4 (AS Neo) and Antisense IL-8 Transfectant in Prostate of Nude Mice

Cell line	Tumorigenicity		Lymph node metastasis	
	Incidence	Median bladder weight (Range) (mg)	Incidence	Median lymph node weight (Range) (mg)
Sense Transfection				
PC3-P	4 / 7	55 (24 - 480)	4 / 7	20 (16 - 28)
PC3-P(Neo)	6 / 9	120 (27-443)	4 / 9	24 (15 - 32)
PC3-P(IL-8)	8 / 8[a]	1,270 (258 - 1,850)[b]	8 / 8[a]	93 (36 -124)[b]
PC3-P(IL-8 Low)	5 / 8	155 (25 – 720)	5 / 8	38 (25 - 69)
PC3-P(IL-8 High)	9 / 9[a]	1,975 (355 - 2,915)[b]	9 / 9[a]	131 (55 - 188)[b]
Antisense Transfection				
PC3M-LN4	5 / 5	1,921 (250 - 3,359)	5 / 5	101 (19 - 155)
PC3M-LN4(AS Neo)	5 / 5	543 (312 - 713)	5 / 5	110 (24 - 122)
PC3M-LN4(AS IL-8)	2 / 8[c,f]	- (33, 36)[d,f]	0 / 8[e]	
PC3M-LN4(AS IL-8 Low)	2 / 8[c,f]	- (31, 39)[d,f]	0 / 8[e]	
PC3M-LN4(AS IL-8 High)	6 / 9	39 (30 - 525)[e]	0 / 9[e]	

[a] $P<0.05$ against PC3-P and PC3-P Neo (chi-squared test)
[b] $P<0.005$ against PC3-P and PC3-P(Neo) (Mann-Whitney statistical comparison)
[c] $P<0.05$
[d] $P<0.0005$ against PC3M-LN4 and PC3M-LN4(As Neo) (Mann-Whitney statistical comparison)
[e] $P<0.0005$ against PC3M-LN4 and PC3M-LN4(As Neo) (chi squared test)
[f] Explants grown in culture, no detectable tumor by immunohistochemistry

Table 2 The mRNA Expression Level, Protein Expression Level and Microvessel Density in Prostate Tumor with PC3-P, PC3-P (Neo), and Sense IL-8 Transfectants (A) and with PC3M-LN4, PC3M-LN4 (AS Neo), and Antisense IL-8 Transfectants (B)

	mRNA expression index[a]				Protein expression index[b]				Microvessel density[c]
Cell line	IL-8	bFGF	VEGF	MMP-9	IL-8	bFGF	VEGF	MMP-9	(per 200 x field)
Sense Transfection									
PC3-P	100	100	100	100	100	100	100	100	40 ± 9
PC3-P(Neo)	117	98	99	119	108	96	105	117	45 ± 6
PC3-P(IL-8)	288	99	94	295	300	100	100	275	51 ± 12[d]
PC3-P(IL-8 Low)	182	100	96	178	215	96	111	192	80 ± 13
PC3-P(IL-8 High)	371	102	96	349	275	104	105	275	91 ± 18[d]
Antisense Transfection									
PC3M-LN4	100	100	100	100	100	100	100	100	100 ± 20
PC3M-LN4(AS Neo)	101	101	97	102	100	102	96	96	104 ± 23
PC3M-LN4(AS IL-8 High)	58	98	94	68	57	102	100	67	47 ± 14[e]

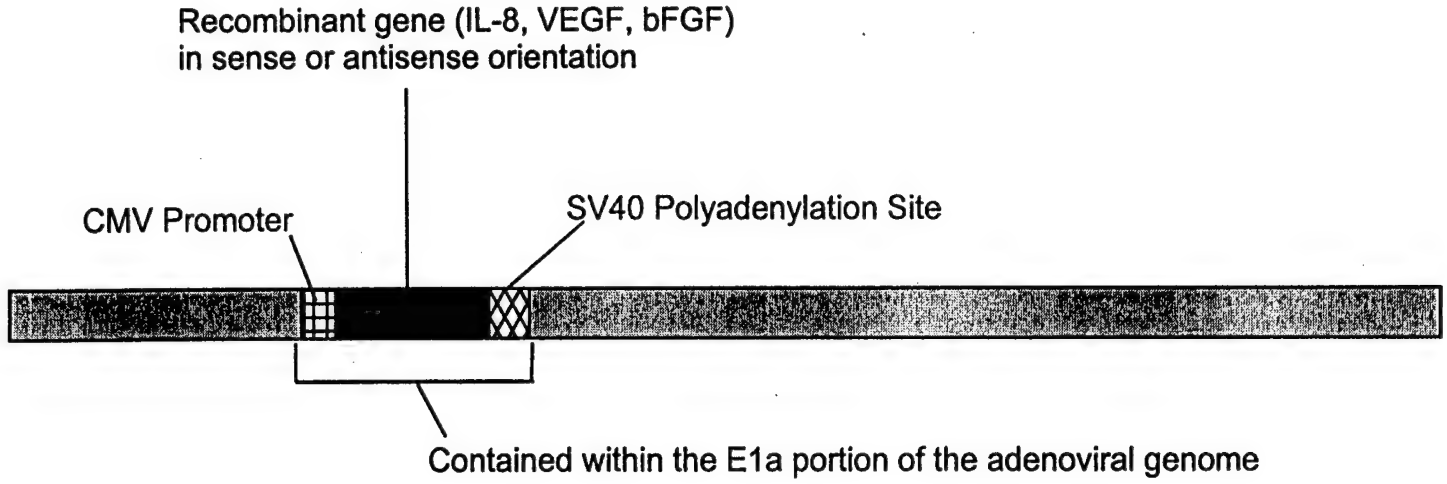
[a]The intensity of the cytoplasmic color reaction was quantified by an image analyzer and compared with maximal intensity of poly d(T) color reaction in each sample. The results were presented as the number of calls for each line with PC3-P (A) and PC3M-LN4 (B) defined as 100.

[b]The intensity of the cytoplasmic immunostaining was quantified by an image analyzer in three different areas of each sample to yield an average measurement and compared with the intensity of the normal epithelial cells of prostate glands and adjusted to the intensity of the cells of the tumors with parental cell line devined as 100.

[c]Microvessel density was expressed as an average number of five highest area identified within a single 200 x field.

[d]P<0.005 against PC3-P and PC3-P(Neo)

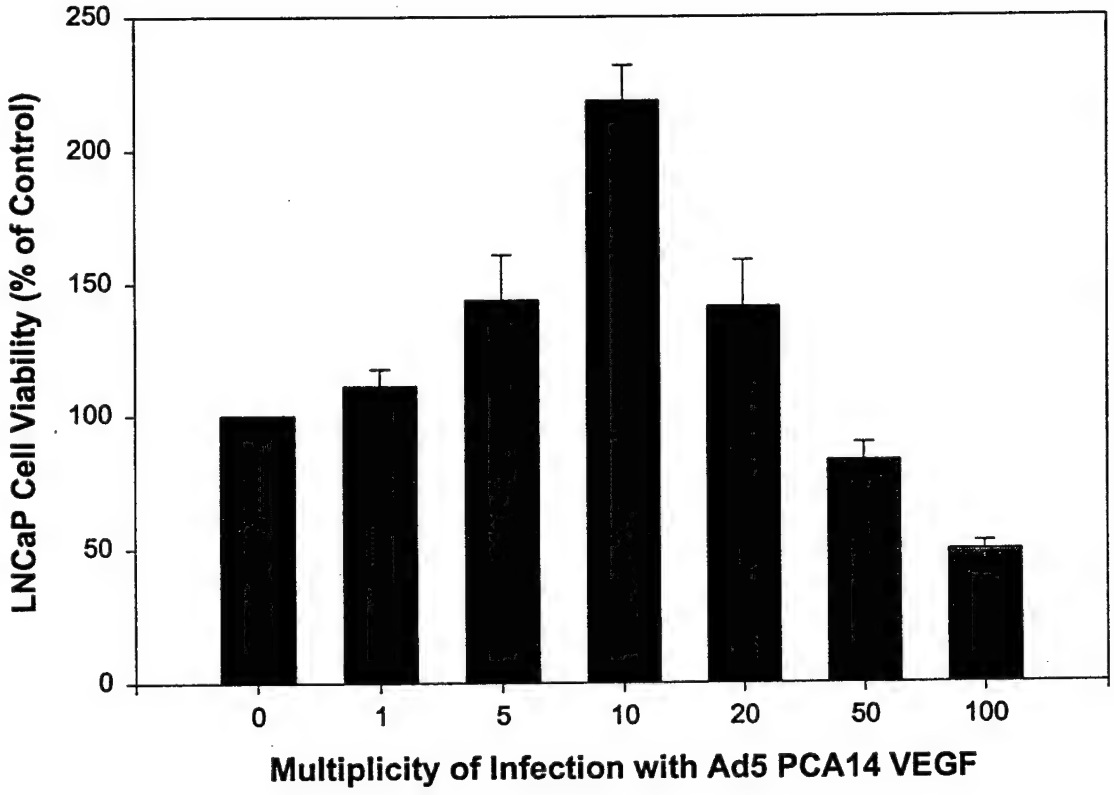
[e]P<0.005 against PC3M-LN4 and PC3M-LN4 and PC3M-LN4(As Neo) (Mann-Whitney statistical comparison)

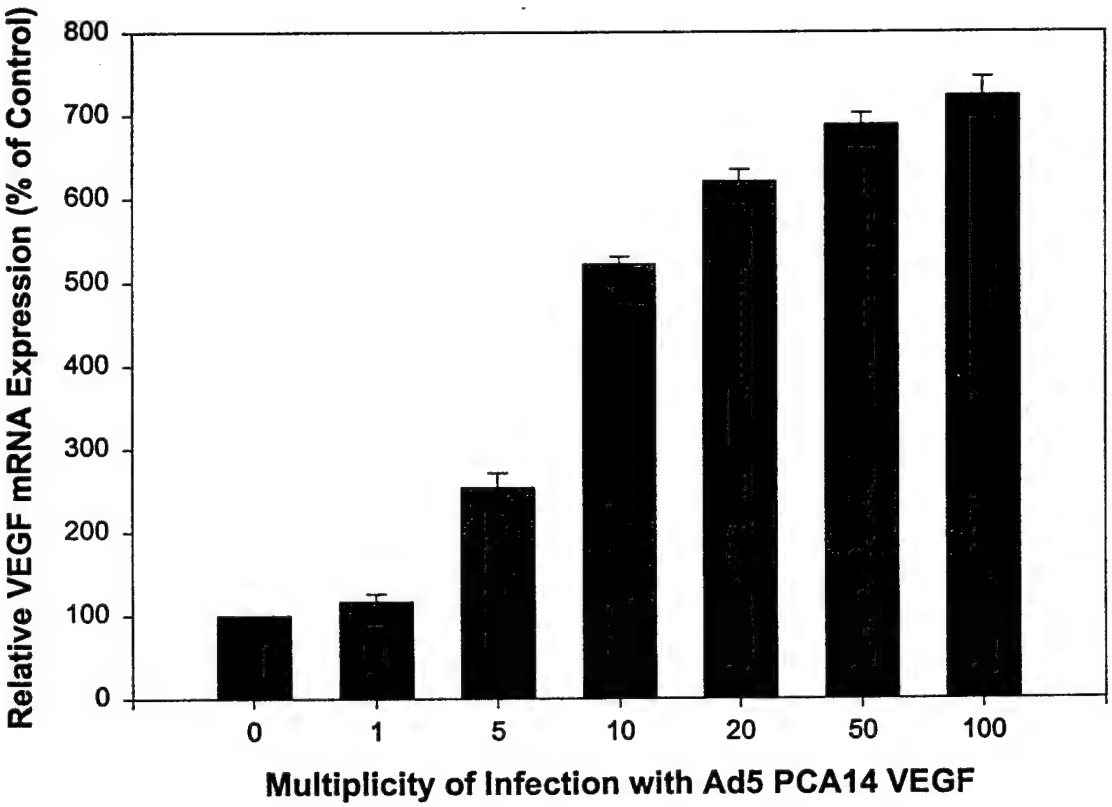


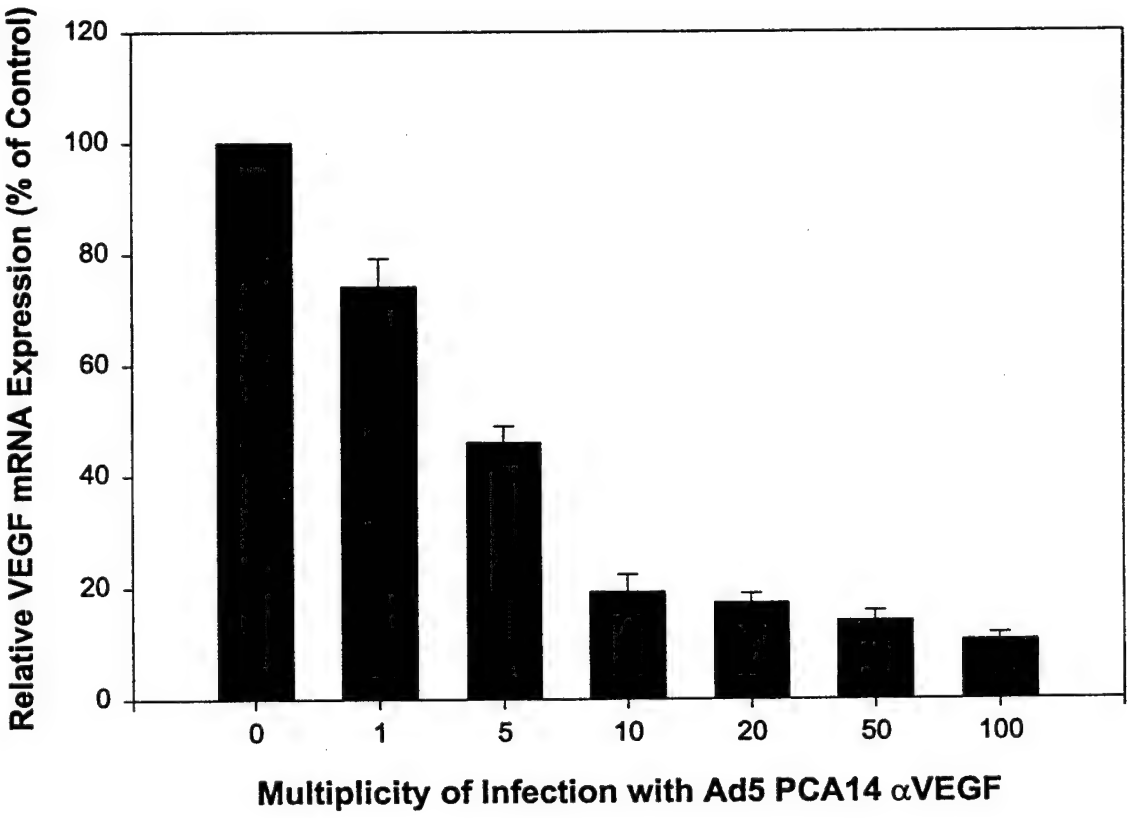
Ad5 PCA-14 Replication Deficient Adenovirus

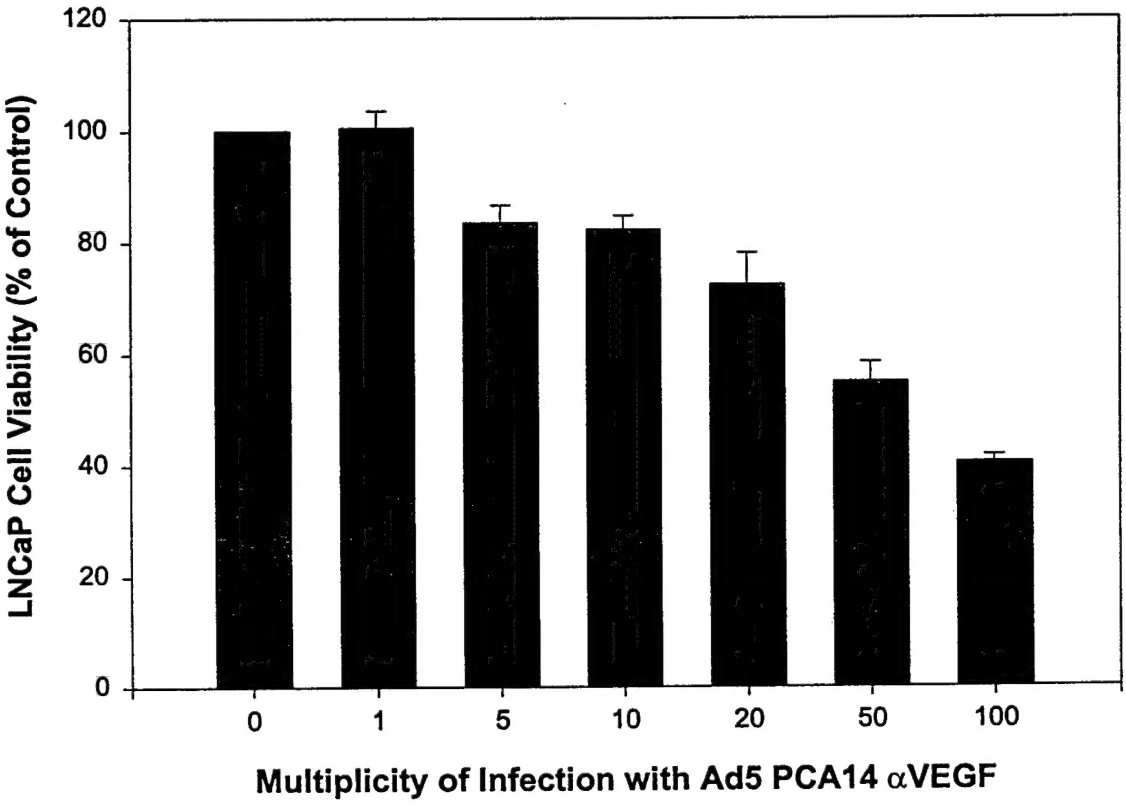
A. Schematic representation of recombinant adenoviral constructs used to modulate angiogenesis factor expression.

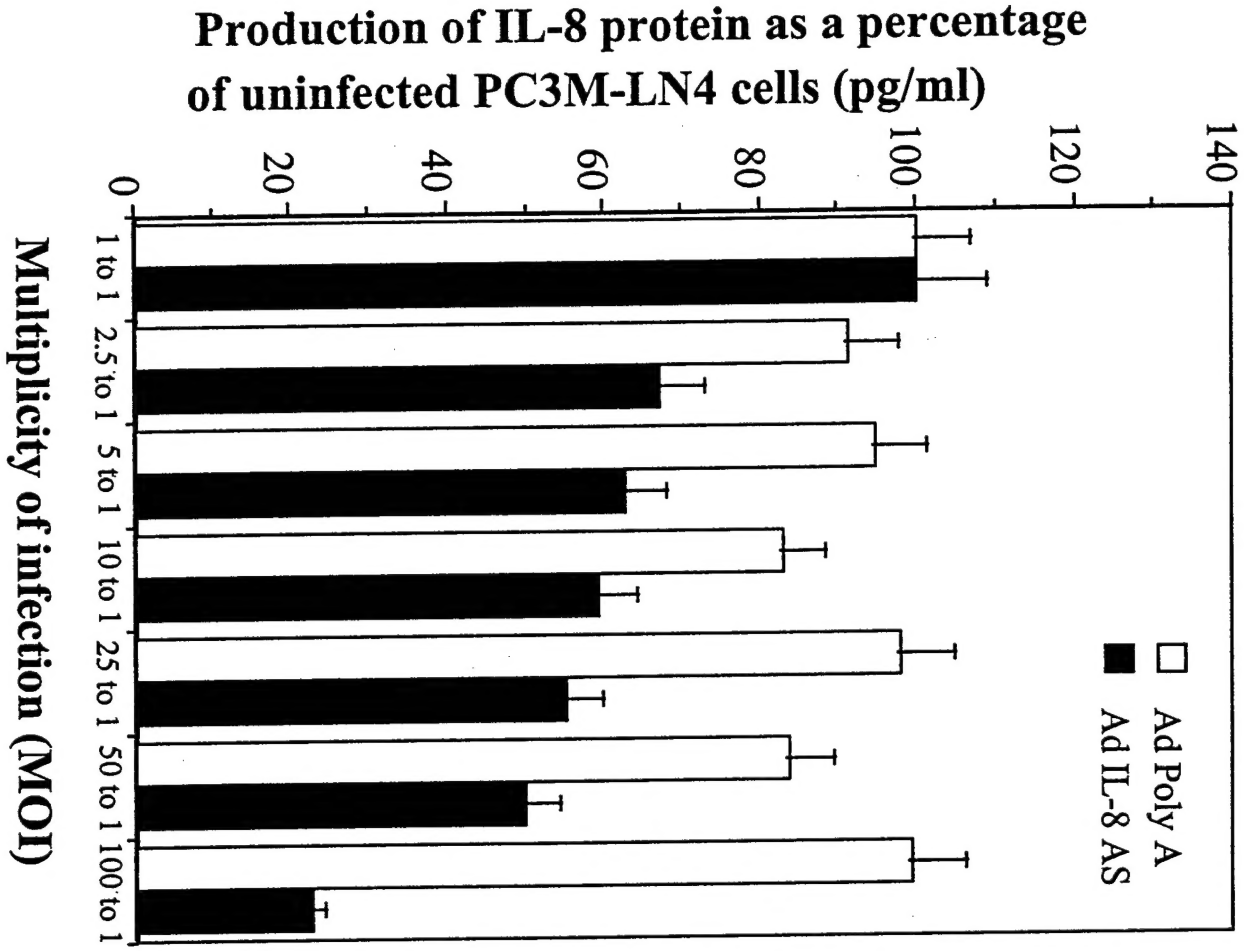
The full length cDNA's for the coding region of interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) were cloned from human total cellular RNA samples using reverse transcriptase-polymerase chain reaction techniques. After the integrity of the cDNA sequences was confirmed using PCR sequencing, the recombinant genes were cloned into the shuttle vector PCA-14. PCA 14 contains Adenovirus 5 sequences from bp 22 to bp 5790, with a deletion of E1 sequences from bp 342 to 3523. A poly cloning site flanked by a CMV promoter and an SV40 polyadenylation site is the point of insertion for the recombinant gene (IL-8, VEGF, or bFGF), in either the sense or antisense orientation. The shuttle vector was then co-transfected with the plasmid JM-17, which contains the remainder of the adenoviral genome, into 293 cells. Recombinant, replication deficient, adenovirus was isolated following homologous recombination, then amplified and titered in 293 cells.


LNCaP Cell Viability (% of Control)
250
200
150
100
50
0
0
1
5
10
20
50
100
Multiplicity of Infection with Ad5 PCA14 VEGF


Relative VEGF mRNA Expression (% of Control)
800
700
600
500
400
300
200
100
0
0
1
5
10
20
50
100
Multiplicity of Infection with Ad5 PCA14 VEGF


Relative VEGF mRNA Expression (% of Control)
120
100
80
60
40
20
0
0
1
5
10
20
50
100
Multiplicity of Infection with Ad5 PCA14 αVEGF


120
100
80
60
40
20
0
LNCaP Cell Viability (% of Control)
0
1
5
10
20
50
100
Multiplicity of Infection with Ad5 PCA14 αVEGF


Production of IL-8 protein as a percentage of uninfected PC3M-LN4 cells (pg/ml)
0
20
40
60
80
100
120
140
1 to 1
2.5 to 1
5 to 1
10 to 1
25 to 1
50 to 1
100 to 1
Multiplicity of infection (MOI)
Ad Poly A
Ad IL-8 AS

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Prostate Cancer

Metastases

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VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) OVEREXPRESSION PREDICTS LYMPH NODE METASTASIS IN CLINICALLY LOCALIZED PROSTATE CANCER. Hiroki Kuniyasu, Patricia Troncoso, Curtis A. Pettaway and Colin P. N. Dinney, Houston, TX (Presented by Dr. Dinney).

INTRODUCTION AND OBJECTIVES: The purpose of this study was to determine whether the steady-state gene expression of VEGF (a potent angiogenic molecule) by clinically localized prostate cancer predicts lymph node metastases.

METHODS: Eighteen formalin-fixed paraffin-embedded archival prostate cancer specimens were examined by a colorimetric in situ hybridization assay for the steady-state mRNA expression of VEGF. The intensity of VEGF staining by the tumor was quantified by image analysis and compared with the expression by normal prostate glandular epithelium. The integrity of the mRNA was determined by poly-d(T)$_{20}$ in situ hybridization.

RESULTS: In 6 of the 18 patients, lymph node metastases were detected at the time of radical prostatectomy. These 6 primary tumors demonstrated a significant increase in VEGF mRNA expression (relative median VEGF intensity 319, range 243-755) compared with the tumors from patients without metastases (median VEGF intensity 138 range 76-342; p= 0.0027). VEGF expression was independent of serum PSA level but correlated with Gleason score.

CONCLUSIONS: VEGF mRNA is overexpressed by high-grade prostate cancer and may contribute to its metastatic potential.

SOURCE OF FUNDING: Robert Wood Foundation.

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Abstract #911- Revised

ASSESSMENT OF METASTASIS-RELATED GENE EXPRESSION IN PROSTATE BIOPSY SPECIMENS: A NOVEL REDICTOR OF PATHOLOGIC STAGE

Hiroki Kuniyasu, Patricia Troncoso, Keiji Inoue, Isaiah J. Fidler, Colin P. N. Dinney, and Curtis A. Pettaway, Houston, TX (Presented by Dr. Pettaway).

INTRODUCTION AND OBJECTIVES: The relative expressions of genes associated with invasion (type IV collagenase, specifically the matrix metalloproteinases MMP-2 and MMP-9), cell cohesion (E-cadherin) and angiogenesis (VEGF) were previously shown to be highly associated with pathologic stage in radical prostatectomy (RP) specimens(J Urol 159:72A, 1998; J Urol 159:291A, 1998). In the present study, we assessed the correlation between the expression of these metastasis-related genes in pretherapy biopsy and RP specimens to determine whether their expression in pretherapy biopsy (BX) specimens could predict pathologic stage after RP and to determine whether such an assessment could be used to select suitable patients for RP.

METHODS: Twenty-one prostate cancer BX specimens obtained from 16 patients (clinical stage T2) and individual tumor foci from the corresponding RP specimens [stage pT2 (6 patients) , pT3 (6), pTany,N+ (4)] were examined using a colorimetric in situ hybridization assay. The mRNA expression levels of collagenase (MMP-2 and MMP-9), E-cadherin, and VEGF as well as the MMP/E-cadherin ratio was compared in BX and RP specimens and analyzed as a predictor of pathologic stage.

RESULTS: MMP-2, MMP-9, E-cadherin, VEGF, and the MMP/ E-cadherin ratio were all highly related to pathologic stage and correlated well between pretherapy BX and RP with the exception of MMP-2. However, the biopsy specimens failed to predict aggressive cancers in 3 of 11 cases. The prediction of pT3 or pTany,N1 cancers (sensitivty 60-70% specificty 67-100%) based upon Bx mRNA expression levels appeared superior to BX Gleason score.

CONCLUSIONS: These results suggest that evaluation of metastasis related gene expression in formalin fixed biopsies is feasible, correlates with
advanced pathologic stage in clinically localized prostate cancers and may provide unique staging information.

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